(51) International Patent Classification ^{6} :	A1	(11) International Publication Number:	WO 98/21938
A01H 5/00, C07K 14/415, 16/16, C12N 1/13, 1/21, 5/10, 15/29, 15/64, 15/82		(43) International Publication Date:	28 May 1998 (28.05.98)

(21) International Application Number: PCT/US97/21336

(22) International Filing Date: 18 November 1997 (18.11.97)

(30) Priority Data:
60/031,040   18 November 1996 (18.11.96)   US
60/061,328   8 October 1997 (08.10.97)   US

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(81) Designated States: AU, CA, JP, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.

(54) Title: GLUTATHIONE-S-CONJUGATE TRANSPORT IN PLANTS

(57) Abstract

The invention includes an isolated DNA encoding a plant GS–X pump polypeptide and an isolated preparation of a plant GS–X pump polypeptide. Also included is an isolated preparation of a nucleic acid which is antisense in orientation to a portion or all of a plant GS–X pump gene. The invention also includes cells, vectors and transgenic plants having an isolated DNA encoding a plant GS–X pump and methods of use thereof. In addition, the invention relates to plant GS–X pump promoter sequences and the uses thereof.

GLUTATHIONE-S-CONJUGATE TRANSPORT IN PLANTS

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BACKGROUND OF THE INVENTION

Animal and plant cells have the capacity to eliminate a diversity of lipophilic toxins from the cytosol following conjugation of the toxin with glutathione (GSH) (Ishikawa et al., 1997, *Bioscience Reports.* 17:189-208; Martinoia *et al.*, 1993, *Nature* 364:247-249; Li *et al.*, 1995, *Plant Physiol.* 107:1257-1268). This process is

15 mediated by the glutathione *S*-conjugate (GS-*X*) pumps which are novel MgATP-dependent transporters that catalyze the efflux of GS-conjugates and glutathione disulfide (GSSG) from the cytosol via the plasma membrane and/or endomembranes. GS-*X* pumps are considered to constitute a terminal phase of xenobiotic detoxification

20 in animals and plants.

The metabolism and detoxification of xenobiotics comprises three main phases (Ishikawa, 1992, *supra*). Phase I is a preparatory step in which toxins are oxidized, reduced or hydrolyzed to introduce or expose functional groups having an appropriate reactivity. Cytochrome P450 monooxygenases and mixed function

25 oxidases are examples of phase I enzymes. In phase II, the activated derivative is

conjugated with GSH, glucuronic acid or glucose. In the case of the GSH-dependent
pathway, S-conjugates of GSH are formed by cytosolic glutathione-S-transferases
(GSTs). In the final phase, phase III, of the GSH-dependent pathway, GS-conjugates
are eliminated from the cytosol by the GS-X pump.

5   The GS-X pump is unique in its exclusive use of MgATP, rather than
preformed transmembrane ion gradients, as a direct energy source for organic solute
transport. Although an understanding of the constituents of GS-X pumps is relevant to
an understanding of the mechanism by which cells combat, for example,
chemotherapeutic agents and herbicides, there has until recently been a paucity of

10   information on the molecular identity of GS-X pumps, particularly in plants.

A 190 kDa membrane glycoprotein encoded by the human multidrug
resistance-associated protein gene (MRP1) has been implicated in the resistance of
small cell lung cancer cell lines to a number of chemotherapeutic drugs (Cole *et al.*,
1992, *Science* 258:1650-1654). This glycoprotein catalyzes the MgATP-dependent

15   transport of leukotriene C_4 and related glutathione-S-conjugates (Leier *et al.*, 1994, *J.
Biol. Chem.* 269:27807-27810; Muller *et al.*, 1994, *Proc. Natl. Acad. Sci. USA*
91:13033-13037; Zamam *et al.*, 1995, *Proc. Natl. Acad. Sci. USA* 92:7690-7694).

MRP1 is a member of the ATP binding cassette (ABC) superfamily of
transporter proteins. Distributed throughout the major taxa, ABC transporters catalyze

20   the MgATP-dependent transport of peptides, sugars, ions and lipophiles across
membranes. ABC transporters comprise one or two copies each of two basic structural
elements, a hydrophobic integral membrane sector containing approximately six
transmembrane α helices and a cytoplasmically oriented ATP-binding domain known
as a nucleotide binding fold (NBF) (Hyde *et al.*, 1990, *Nature* 346:362-365; Higgins,

25   1995, *Cell* 82:693-696). The NBFs are a diagnostic feature of ABC transporters and
are 30% identical between family members over a span of about 200 amino acid
residues, having two regions known as a Walker A and a Walker B box (Walker *et al.*,
1992, *EMBO J.* 1:945-951), and also having an ABC signature motif (Higgins, 1995,
supra).

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ABC family members in eukaryotes include mammalian P-glycoproteins (P-gps or MDRs), some of which are implicated in drug resistance and others in lipid translocation (Ruetz et al., 1994, Cell 77:1071-1081), the pleiotropic drug resistance protein (PDR5) and STE6 peptide mating pheromone transporter of

5   yeast, the cystic fibrosis transmembrane conductance regulator (CFTR) Cl⁻ channel, the malarial Plasmodium falciparum chloroquine transporter (PFMDR1) and the major histocompatibility (MHC) transporters responsible for peptide translocation and antigen presentation (Balzi et al., 1994, J. Bioenerg. Biomemb. 27:71-76; Higgins, 1995, supra).

10   Sequence comparisons between MRP1 and other ABC transporters reveal two major subgroups among these proteins (Cole et al., 1992, supra; Szczypka et al., 1994, J. Biol. Chem. 269:22853-22857). One subgroup comprises MRP1, the Saccharomyces cerevisiae cadmium factor (YCF1) gene, the Leishmania P-glycoprotein-related molecule (Lei/PgpA) and the CFTRs. The other subgroup

15   comprises the multiple drug resistance proteins (MDRs), MHC transporters and STE6.

The invention described herein relates to bioremediation (specifically phytoremediation), plant responses to herbicides, plant-pathogen interactions and plant pigmentation.

With respect to bioremediation, the massive global expansion in

20   industrial and mining activities during the last two decades together with changes in agricultural practices, has markedly increased contamination of groundwaters and soils with heavy metals. Indeed, it is estimated that the annual toxicity of metal emissions exceeds that of organics and radionuclides combined (Nriagu et al., 1988, Nature 333:1340138). Since soil and water contamination results in the uptake of heavy

25   metals and toxins by crop plants, and eventually humans, there remains a need for a means of manipulating the ability of a plant to sequester compounds from the soil in order to better manage soil detoxification through bioremediation using native species or genetically engineered organisms.

Regarding herbicides, these compounds are generally low molecular weight, lipophilic compounds that readily penetrate cells in a passive manner. Having entered cells, herbicides inhibit plant-specific processes such as photosynthetic electron transport (*e.g.*, atrazine, chlortoluron) or the biosynthesis of essential amino acids (*e.g.*,

5   glyphosate, chlorsulfuron or phosphotricine), porphyrins (*e.g.*, acidofluorfen), carotenoids (*e.g.*, norflurazon), fatty acids (*e.g.*, diclofop) or cellulose (*e.g.*, dichlobenil) (Boger *et al.*, 1989, *Target Sites of Herbicide Action*, CRC Press, Boca Raton, FL; Devine *et al.*, 1993, *Physiology of Herbicide Action*, Prentice Hall, Englewood Cliffs, NJ). Plants that are naturally tolerant of certain herbicides either

10   contain a cellular target that does not interact with the herbicide, have efficient systems for inactivation of the herbicide, or have a high capacity for excluding or eliminating the herbicide from the target.

Herbicide metabolism comprises the three phases described above for general xenobiotic metabolism. The first two phases (the first being oxidation and

15   hydrolysis and the second being conjugation with GSH or glucose) contribute to detoxification by decreasing the intrinsic biochemical activity of the herbicide and/or by increasing its hydrophilicity. These two phases render the herbicide less mobile in the plant. The third phase (compartmentation) is often critical for sustained detoxification since the conjugates themselves may interfere with metabolism. For

20   example, the herbicide synergist tridiphane, is converted to its corresponding GS-conjugate in plants to generate a potent inhibitor of atrazine metabolism. (Lamoureux *et al.*, 1986, *Pestic. Biochem. Physiol.* 26:323-342).

Likewise, and more generally, GS-conjugates of any given herbicide would be expected to act as end-product inhibitors of GSTs and thereby impair long-

25   term detoxification unless they are removed from the intracellular compartment, usually the cytosol, in which they are formed. Since the vacuolar GS-*X* pump of plants is known to transport several GS-herbicide conjugates, for example, those of the chloroacetanilide herbicides (metolachlor) and triazines (simetryn) (Martinoia *et al.*, 1993, *supra*; Li *et al.*, 1995, *supra*), there is a long felt need for a knowledge of the

molecular identity of this transporter or family of transporters. Such knowledge will
enable the development of new strategies for increasing or decreasing the resistance of
plants to herbicides.

5   With regard to plant-pathogen interactions, a key event in the disease
resistance response of legumes is the rapid and localized accumulation of isoflavonoid
phytoalexins. The majority of the research on plant-pathogen interactions has centered
on the enzymology and molecular biology of the isoflavonoid biosynthetic pathway
(Dixon *et al.*, 1995, *Physiol. Plant* 93:385). However, the mechanism and sites of
intracellular accumulation of these compounds is not understood. Since many

10   isoflavonoid phytoalexins are as toxic to the host plant as they are to its pathogens, the
discovery of the molecular mechanism by which these compounds are sequestered
within a plant is crucial to the development of plants with increased pathogen
resistance.

          With regard to plant pigmentation, functional analyses of the maize

15   gene, *Bronze-2*, which participates in anthocyanin pigment biosynthesis, suggest that
one of the endogenous substrates for the plant vacuolar GS-*X* pump are anthocyanin-
GS conjugates (Marrs *et al.*, 1995, *Nature*, 375:397-400). Anthocyanins share a
common biosynthetic origin and core structure based on cyanidin-3-glucoside. It is
through the species-specific decoration of cyanidin-3-glucoside by hydroxylation,

20   methylation, glucosylation and acylation that the wide spectrum of red, blue and purple
colors in the vacuoles of flowers, fruits and leaves is produced. The molecular nature
of the plant GS-*X* pump which mediates transport of anthocyanin-GS conjugates was
not known in the art until the present invention. There remains a need to determine the
molecular nature of the GS-*X* pump responsible for transport of anthocyanin-GS

25   conjugates in order that plant coloration may be manipulated at the molecular level.
          The present invention satisfies the aforementioned needs.

BRIEF SUMMARY OF THE INVENTION

The invention includes an isolated DNA encoding a plant GS-*X* pump polypeptide. In one aspect, the isolated DNA is selected from the group consisting of DNA comprising *AtMRP1* and *AtMRP2*, and any mutants, derivatives, homologs and fragments thereof encoding GS-X pump activity.

The invention also includes an isolated preparation of a polypeptide comprising a plant GS-*X* pump. In one aspect of this aspect of the invention, the polypeptide is selected from the group consisting of AtMRP1, AtMRP2, and any mutants, derivatives, homologs and fragments thereof having GS-*X* pump activity.

Also included in the invention is a recombinant cell comprising an isolated DNA encoding a plant GS-*X* pump polypeptide. In one aspect, the cell is selected from the group consisting of a prokaryotic cell and a eukaryotic cell.

Further included in the invention is a vector comprising an isolated DNA encoding a plant GS-*X* pump polypeptide.

The invention also includes an antibody specific for a plant GS-*X* pump polypeptide.

In addition, an isolated preparation of a nucleic acid which is in an antisense orientation to all or a portion of a plant GS-*X* pump gene is included in the invention and a cell and a vector comprising this isolated preparation of a nucleic acid are further included.

The invention also relates to a transgenic plant, the cells, seeds and progeny of which comprise an isolated DNA encoding a plant GS-*X* pump.

In addition, the invention relates to a transgenic plant, the cells, seeds and progeny of which comprise an isolated preparation of a nucleic acid which is in an antisense orientation to all or a portion of a plant GS-*X* pump gene.

Further, there is included a transgenic plant, the cells, seeds and progeny of which comprise an isolated DNA encoding YCF1, or any mutants, derivatives, homologs and fragments thereof having YCF1 activity.

The invention further relates to an isolated DNA comprising a plant GS-X pump promoter sequence. In one aspect, the promoter sequence is selected from the group consisting of an *AtMRP1* and an *AtMRP2* promoter sequence.

Also included in this aspect of the invention is a cell and a vector comprising an isolated DNA comprising a plant GS-X plant promoter sequence.

The invention additionally relates to a transgenic plant, the cells, seeds and progeny of which comprise a transgene comprising an isolated DNA comprising a GS-X pump promoter sequence, wherein the GS-X pump promoter sequence is selected from the group consisting of an *AtMRP1*, an *AtMRP2* and a *YCF1* promoter sequence. The promoter sequence may also have operably fused thereto a reporter gene.

There is also included in the invention a method of identifying a compound capable of affecting the expression of a plant GS-X gene. The method comprises providing a cell comprising an isolated DNA comprising a plant GS-X pump promoter sequence having a reporter sequence operably linked thereto, adding to the cell a test compound, and measuring the level of reporter gene activity in the cell, wherein a higher or a lower level of reporter gene activity in the cell compared with the level of reporter gene activity in a cell to which the test compound was not added, is an indication that the test compound is capable of affecting the expression of a plant GS-X pump gene.

In addition, the invention relates to a method of removing xenobiotic toxins from soil. The method comprises growing in the soil a transgenic plant of comprising an isolated DNA encoding a GS-X pump.

Also included is a method of removing heavy metals from soil comprising growing in the soil a transgenic plant of comprising an isolated DNA encoding a GS-X pump.

The invention further relates to a method of generating a transgenic pathogen resistant plant comprising introducing to the cells of the plant an isolated DNA encoding a GS-X pump, wherein the pump is capable of transporting glutathionated isoflavonoid alexins into the cells of the plant.

Additionally, there is included a method of manipulating plant pigmentation comprising modulating the expression of a GS-*X* pump protein in the plant, wherein the GS-*X* pump protein is selected from the group consisting of AtMRP1, AtMRP2 and YCF1.

The invention also relates to a method of alleviating oxidative stress in a plant comprising introducing into the cells of the plant DNA encoding a GS-*X* pump, wherein the DNA is selected from the group consisting of DNA encoding AtMRP1, AtMRP2 and YCF1.

Further included is a method of manipulating the expression of a gene in a plant cell. The method comprises operably fusing a GS-*X* pump promoter sequence to the DNA sequence encoding the gene to form a chimeric DNA, and generating a transgenic plant, the cells of which comprise the chimeric DNA, wherein upon activation of the GS-*X* pump promoter sequence, the expression of the gene is manipulated.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a series of graphs depicting differential sensitivities of DYT165 cells (wild type, Figure 1A) and DTY167 cells (*ycf1*Δ mutant, Figure 1B) to growth inhibition by 1-chloro-2,4-dinitrobenzene (CDNB). Cells were grown at 30°C for 24 hours to an $OD_{600\,nm}$ of approximately 1.4 in YPD medium before inoculation of aliquots into 15 ml volumes of the same medium containing 0-60 μM CDNB. $OD_{600\,nm}$ was measured at the times indicated.

Figure 2 is a graph depicting the time course of [^{3}H]DNP-GS uptake by vacuolar membrane vesicles purified from DTY165 and DTY167 cells. Uptake was measured in the absence (-MgATP) or presence of 3 mM MgATP (+MgATP) in reaction media containing 66.2 μM [^{3}H]DNP-GS, 10 mM creatine phosphate, 16 units/ml creatine kinase, 50 mM KCl, 0.1% (w/v) bovine serum albumin, 400 mM sorbitol, and 25 mM Tris-MES (pH 8.0) at 25°C. Values shown are means ± S.E. (*n* = 3).

Figure 3 is a series of graphs depicting the kinetics of uncoupler-insensitive [³H]DNP-GS uptake by vacuolar membrane vesicles purified from DTY165 and DTY167 cells. **Figure 3A:** MgATP concentration-dependence of uncoupler-insensitive uptake. **Figure 3B:** DNP-GS concentration-dependence of MgATP-dependent uncoupler-insensitive uptake. The MgATP concentration-dependence of uptake was measured with 66.2 µM [³H] DNP-GS. The DNP-GS concentration-dependence of uptake was measured with 3 mM MgATP. Uptake was allowed to proceed for 10 minutes in standard uptake medium containing 5 µM gramicidin D. The kinetic parameters for vacuolar membrane vesicles purified from DTY165 cells were $K_{m(MgATP)}$ 86.5 ± 29.5 µM, $K_{m(DNP-GS)}$ 14.1 ± 7.4 µM, $V_{max(MgATP)}$ 38.4 ± 5.6 nmol/mg/10 minutes, $V_{max(DNP-GS)}$ 51.0 ± 6.3 nmol/mg/10 minutes. The lines of best fit and kinetic parameters were computed by nonlinear least squares analysis (Marquardt, 1963, *J. Soc. Ind. Appl. Math.* 11:431-441). Values shown are means ± S.E. (*n* = 3).

Figure 4 is a series of graphs depicting sucrose density gradient fractionation of vacuolar membrane-enriched vesicles prepared from DYT165 cells. One ml (1.1 mg protein) of partially purified vacuolar membrane vesicles derived from vacuoles prepared by the Ficoll flotation technique were applied to a linear sucrose density gradient (10-40%, w/v) and analyzed for protein (**Figure 4A**), α-mannosidase activity (**Figure 4B**), V-ATPase activity (**Figure 4C**), and MgATP-dependent, uncoupler-insensitive [³H]DNP-GS uptake (**Figure 4D**). [³H]DNP-GS uptake and enzyme activity were assayed as described herein in Table 4 and the accompanying text.

Figure 5 includes a graph (**Figure 5A**) depicting the effect of transformation with pYCF1-HA or pRS424 on MgATP-dependent, uncoupler-insensitive [³H]DNP-GS uptake by vacuolar membranes purified from DTY165 and DTY167 cells. Uptake was measured in standard uptake medium containing 66.2 µM [³H]DNP-GS and 5 µM gramicidin D. Also shown (**Figure 5B**) is an image of a gel depicting immunoreaction of vacuolar membrane proteins prepared from pYCF1-HA-

transformed and pRS424-transformed DTY165 and DTY167 cells with mouse

monoclonal antibody raised against the 12CA5 epitope of human influenza

hemagglutinin. All lanes were loaded with 25 μg of delipidated membrane protein and

subjected to SDS-polyacrylamide gel electrophoresis and Western analysis as described

5 herein. The M_r of YCF1-HA (boldface type) and the positions of the M_r standards are

indicated on the figure.

  Figure 6 is a series of graphs depicting transformation with pYCF1-HA

(Figure 6A) or pRS424 (Figure 6B) on the sensitivity of DTY167 cells to growth

retardation by CDNB. Cells were grown at 30°C for 24 hours to an $OD_{600\ nm}$ of

10 approximately 1.4 in AHC medium (Kim *et al.*, 1994, *Proc. Natl. Acad. Sci. USA*

91:6128-6132) before inoculation of aliquots into 15 ml volumes of the same medium

containing 0-60 μM CDNB. $OD_{600\ nm}$ was measured at the times indicated.

  Figure 7 is a series of photomicrographs of DTY165 (Figures 7A and

7C) and DTY167 cells (Figures 7B and 7D) after incubation with monochlorobimane.

15 Cells were grown in YPD medium for 24 hours at 30°C and 100 μl aliquots of cell

suspensions were transferred into 15 ml volumes of fresh YPD medium containing 100

μM monochlorobimane. After incubation for 6 hours, the cells were washed and

examined in fluorescence (Figures 7C and 7D) or Nomarski mode (Figures 7A and

7B) as described herein.

20 Figure 8 is a series of graphs depicting uptake of Cd^{2+} into vacuolar

membrane vesicles purified from DTY165 and DTY167 cells. Uptake of $^{109}Cd^{2+}$ by

DTY165 membranes (Figure 8A) or DTY167 membranes (Figure 8B) was measured

in the absence of MgATP plus (O) or minus GSH (1 mM) (□) or in the presence of

MgATP (3 mM) plus (●) or minus (■) GSH. $^{109}Cd_2SO_4$ and gramicidin-D were

25 added at concentrations of 80 μM and 5 μM, respectively. Figure 8C: Rate of $^{109}Cd^{2+}$

uptake by DTY165 membranes plotted as a function of the total concentration of Cd^{2+}

($[Cd^{2+}]_{total}$) added to uptake medium containing 1 mM GSH, 3 mM MgATP and 5 μM

gramidicin-D. Values shown are means ± SE ($n = 3$-6).

Figure 9 is a series of graphs depicting purification of cadmium glutathione complexes by gel-filtration (**Figure 9A**) and anion-exchange chromatography (**Figures 9B and 9C**). Twenty mM $^{109}Cd_2SO_4$ was incubated with 40 mM GSH at 45°C for 24 hours and the mixture was chromatographed on Sephadex G-

5 15 to resolve a high molecular weight ^{109}Cd-labeled component (*HMW-^{109}Cd.GS*) from a low molecular weight component (*LMW-^{109}Cd.GS*) (**Figure 9A**). The peaks corresponding to *HMW-^{109}Cd.GS* and *LMW-^{109}Cd.GS* were then chromatographed on Mono-Q and eluted with a linear NaCl gradient (---) (**Figure 9B and 9C**). ^{109}Cd (cpm $\times 10^{-3}$) was determined on 5 µl aliquots of the column fractions by liquid scintillation

10 counting.

Figure 10 is a series of graphs depicting the kinetics of MgATP-dependent, uncoupler-insensitive ^{109}Cd.GS$_2$ (*HMW-^{109}Cd.GS*, **Figure 10A**) and ^{109}Cd.GS (*LMW-^{109}Cd.GS*, **Figure 10B**) uptake. DNP-GS was added at the concentrations (µM) indicated to DTY165 membranes (●,○,■,□,△) or DTY167

15 membranes (◊). A secondary plot of the apparent Michaelis constants for Cd.GS$_2$ uptake (K_m^{app}/Cd.GS$_2$) as a function of DNP-GS concentration is shown (**Figure 10C**). The kinetic parameters for Cd.GS$_2$ transport by DTY165 membranes were K_m, 39.1 ± 14.1 µM, V_{max}, 157.2 ± 30.4 nmol/mg/10 minutes and $K_{i(DNP-GS)}$, 11.3 ± 2.1 µM. Kinetic parameters were computed by nonlinear least squares analysis (Marquardt,

20 1963, *supra*). Values shown are means ± SE ($n = 6$).

Figure 11 is a graph depicting matrix-assisted laser desorption mass spectrometry (MALD-MS) of *HMW-Cd.GS*. MALD-MS was performed on Sephadex G-15-, Mono-Q-purified *HMW-Cd.GS* as described herein. The molecular structure inferred from a mean m/z ratio of 725.4 ± 0.7 ($n = 9$) and average Cd.GS stoichiometry

25 of 0.5 [*bis*(glutathionato)cadmium, Cd.GS$_2$, molecular weight 724.6 Da] is shown.

Figure 12 is an image of a gel depicting induction of *YCF1* expression and YCF1-dependent Cd.GS$_2$ and DNP-GS transport by pretreatment of DTY165 cells with CdSO$_4$ (Cd^{2+}, 200 µM) or CDNB (150 µM) for 24 hours. *YCF1*-specific mRNA and 18S rRNA were detected in the total RNA extracted from control or pretreated

cells (10 μg/lane) by RNase protection. Uptake of ^{109}Cd.GS$_2$ (50 μM) or [^{3}H]DNP-GS
(66.2 μM) by vacuolar membrane vesicles was measured in standard uptake medium
containing 5 μM gramicidin-D. Values shown are means ± SE ($n = 3$).

Figure 13A and 13B is the sequence of *AtMRP2* cDNA (SEQ ID
NO:1). Lower case letters correspond to 5'- and 3'-untranslated regions (UTRs).

Figure 14A-D is the genomic sequence of *AtMPR2* (SEQ ID NO:2).
Lower case letters at the beginning and end of the sequence correspond to 5'- and 3'-
UTRs, respectively; lower case letters nested within the sequence correspond to
introns.

Figure 15 is the deduced amino acid sequence of AtMRP2 (SEQ ID
NO:3).

Figure 16A and 16B is the sequence of *AtMRP1* cDNA (SEQ ID
NO:4). Lower case letters correspond to 5'- and 3'-UTRs.

Figure 17A-D is the genomic sequence of *AtMRP1* (SEQ ID NO:5).
Lower case letters at the beginning and end of the sequence correspond to 5'- and 3'-
UTRs, respectively; lower case letters nested within the sequence correspond to
introns.

Figure 18 is the deduced amino acid sequence of AtMRP1 (SEQ ID
NO:6).

Figure 19 is a series of graphs depicting the time course and
concentration-dependence of DNP-GS uptake in *AtMRP1*-transformed yeast. **Figure
19A** is a graph depicting the time course of [^{3}H]DNP-GS uptake by membrane vesicles
purified from pYES3-*AtMRP1*-transformed or pYES3-transformed DTY168 cells.
MgATP-dependent uptake was measured in reaction media containing 61.3 μM
[^{3}H]DNP-GS, 5 μM gramicidin-D, 10 mM creatine phosphate, 16 units/ml creatine
kinase, 50 mM KCl, 1 mg/ml bovine serum albumin, 400 mM sorbitol and 25 mM
Tris-Mes (pH 8.0) at 25°C. Values shown are means ± SE (n = 3). **Figure 19B** is a
graph depicting concentration dependence of MgATP-dependent, uncoupler-insensitive
uptake of [^{3}H]DNP-GS by membrane vesicles purified from pYES3-*AtMRP1*-

transformed DTY168 cells. Uptake was allowed to proceed for 10 minutes in standard

uptake medium containing 5 μM gramicidin-D. The kinetic parameters for uptake

were $K_{m(DNP-GS)}$ 49.7 ± 15.4 μM, V_{max} 6.0 ± 1.7 nmol/mg/10 minutes. The lines of best

fit and kinetic parameters were computed by nonlinear least squares analysis

5   (Marquardt, 1963, supra). Values shown are means ± SE (n = 3).

Figure 20 is a series of graphs depicting sensitivity of MgATP-

dependent, uncoupler-insensitive [³H]DNP-GS uptake by membrane vesicles purified

from pYES3-*AtMRP1*-transformed and pYES3-transformed DTY168 cells. Uptake

was measured for 10 minutes in standard uptake medium containing the indicated

10   concentrations of vanadate. In **Figure 20A**, there is a graph depicting total MgATP-

dependent, uncoupler-insensitive [³H]DNP-GS uptake by membrane vesicles purified

from pYES3-*AtMRP1*-transformed and pYES-transformed DTY168 cells. In **Figure**

20B, there is a graph depicting AtMRP1-dependent uptake. I_{50} (exclusive of

uninhibitable AtMRP1-independent component) = 8.3 ± 3.2 μM. Values shown are

15   means ± SE (n = 3).

Figure 21 is a series of graphs depicting the hydropathy alignment of

AtMRP2, AtMRP1, *S. cerevisiae* YCF1 (ScYCF1), human MRP1 (HmMRP1) and rat

cMOAT (RtCMOAT).

Figure 22 is a diagram depicting domain comparisons between

20   AtMRP1, ScYCF1, HmMRP1, RtCMOAT, rabbit EBCR (RbEBCR) and HmCFTR.

The domains indicated are the N-terminal extension (NH₂), first and second sets of

transmembrane spans (TM1 and TM2, respectively), first and second nucleotide

binding folds (NBF1 and NBF2, respectively), putative CFTR-like regulatory domain

(R), and the C-terminus (COOH).

25   **Figure 23** is the promoter sequence of the *Arabidopsis AtMRP1* gene

(SEQ ID NO:7). Discrete elements which are present in the promoter sequence are

indicated in boldface letters.

Figure 24 is the promoter sequence of the *Arabidopsis AtMRP2* gene (SEQ ID NO:8). Discrete elements which are present in the promoter sequence are indicated in boldface letters.

Figure 25 is a graph depicting MgATP-dependence of [^{3}H]medicarpin

5 uptake by vacuolar membrane vesicles before (Medicarpin/GSH) and after maize GST-mediated conjugation with GSH (Medicarpin-GS). [^{3}H]medicarpin or [^{3}H]medicarpin-GS was added at a concentration of 65 μM. MgATP was either omitted (-MgATP) or added at a concentration of 3 mM (+MgATP). Values shown are means ± SE (n = 3).

Figure 26 is a graph depicting concentration dependence of MgATP-

10 dependent, uncoupler-insensitive [^{3}H]medicarpin-GS uptake into vacuolar membrane vesicles. Uptake was allowed to proceed for 20 minutes in standard uptake medium containing 3 mM MgATP and 5 μM gramicidin D. The kinetic parameters were K_m 21.5 ± 15.5 μM and V_{max} 77.8 ± 23.3 nmol/mg/20 minutes. Values shown are means ± SE (n = 3).

15 Figure 27 is a series of graphs depicting concentration-dependence of MgATP-dependent, uncoupler-insensitive C$_3$G-GS, IAA-GS and ABA-GS uptake by vacuolar membrane vesicles purified from *V. radiata* (Figure 27A) and *Z. mays* (Figure 27B). Uptake was allowed to proceed for 10 minutes in reaction medium containing 50 μM GS-conjugate, 400 mM sorbitol, 3 mM MgATP, 50 mM KCl, 0.1%

20 (w/v) BSA, 5 μM gramicidin-D and 25 mM Tris-Mes (pH 8.0) at 25°C. Values shown are means ± SE (*n* = 3).

Figure 28 is an image of a photograph depicting the growth of wild type (WT) and *YCF1* transgenic *Arabidopsis* (YCF1) seeds on media containing CdSO$_4$ (200 μM) or 1-chloro-2,4-dinitrobenzene (CDNB, 25 μM). Transgenic plants were

25 generated as described herein.

DETAILED DESCRIPTION OF THE INVENTION

The invention is based upon the molecular identification of a new class of membrane transporter in yeast and plants, the GS-*X* pump. As a result of the present

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invention, new insights into the membrane transport phenomena associated with heavy

metal tolerance, herbicide detoxification, plant-pathogen interactions, plant responses

to (phyto)hormones, plant pigmentation and bioremediation are evident. These insights

provide a means, as is evident from the description of the present invention, for the

5   manipulation of plants and the cells thereof, to affect heavy metal tolerance, herbicide

detoxification, plant-pathogen interactions, plant responses to (phyto)hormones, plant

pigmentation and bioremediation.

The process of "storage excretion" is a necessity for plants. Whereas

mammals have the option of excreting GS-conjugates to the extracellular medium for

10   elimination by the kidneys, plants are nearly totally reliant on the sequestration of

noxious compounds in the central vacuole, which frequently accounts for 40-90% of

total intracellular volume. Due to the virtual absence of specialized excretory organs

and the presence of massive vacuoles in plants, a process (intracellular

compartmentation) that is probably only an intermediate step in the elimination of

15   xenobiotics from the cytosol of mammalian cells, is believed to constitute a terminal

phase of detoxification in plants.

The data which are described herein establish that the yeast gene *YCF1*

and two plant homologs of *YCF1*, *AtMRP1* and *AtMRP2*, isolated from *Arabidopsis*

thaliana, each encode a vacuolar GS-X pump. The data further establish that the GS-X

20   pump participates in herbicide metabolism (exemplified by organic xenobiotic

transport), heavy metal sequestration (exemplified by cadmium transport), plant-

pathogen interactions (exemplified by vacuolar uptake of medicarpin), plant cell

pigmentation (exemplified by transport of glutathionated anthocyanins) and plant

hormone metabolism (exemplified by the transport of glutathionated auxins).

25   The plant *AtMRP1* and *AtMRP2* gene products use MgATP as an energy

source for the transport of glutathionated derivatives of both endogenous and

exogenous compounds in plants and thus, the discovery of these genes in the present

invention is important at three levels. The identification of these genes and their

encoded products represents the first identification of ABC transporters in plants for

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which a biochemical function is defined. The discovery establishes, contrary to the prevailing chemiosmotic model for solute transport in plants, that many energy-dependent solute transport processes in plants are not driven by a transmembrane H+ electrochemical potential difference. Further, the identification and isolation of these

5   genes and their encoded products permits a plant element, critical for removal of compounds from the cytosol that can form glutathionine S-conjugates, to be manipulated.

It has been discovered in the present invention that two plant genes, *AtMRP1* and *AtMRP2*, are the structural and functional homologs of the gene encoding

10 yeast YCF1. Proteins encoded by plant *AtMRP1* and *AtMRP2* thus represent a new subclass of ATP binding cassette transporters.

It has been further discovered in the present invention that the yeast YCF1 protein, a GS-*X* pump, is capable of MgATP-energized transport of organic GS-conjugates and of MgATP-energized transport of cadmium upon complexation with

15 GSH. In addition, when plants have introduced into the cells thereof the *YCF1* gene (a transgenic plant comprising *YCF1*), expression of *YCF1* therein confers upon the plants resistance to both inorganic and organiC xenobiotics exemplified by cadmium and 1-chloro-2,4-dinitrobenzene, respectively.

Also discovered in the present invention is the fact that *AtMRP1* and

20 *AtMRP2*, when expressed in a strain of yeast which is deficient in YCF1, can substitute for *YCF1* as a GS-*X* pump. In addition, transformation of plants by *YCF1* confers upon the plant properties which are characteristic of *YCF1* gene expression. Thus, it appears that *YCF1* and the *AtMRP* genes are essentially functionally interchangeable.

In addition, there is provided as part of the invention the

25 promoter/regulatory sequences which control expression of the plant *AtMRP1* and *AtMRP2* genes of the invention. These promoter sequences are useful for the identification of compounds which affect expression of thEse genes in plants and for conferring on other genes the ability to respond to factors that modulate *AtMRP1* and/or *AtMRP2* expression.

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Further discovered in the present invention is the fact that the plant GS-
X pump serves to facilitate the vacuolar storage of antimicrobial compounds induced
following the hypersensitive response to fungal pathogens in the healthy cells
surrounding fungally-induced lesions. Such a process is believed to limit the spread of
5   tissue damage by limiting propagation of the pathogen and spatially delimiting the
toxic action of the phytoalexin itself.

Ascription of specific enzymic and regulatory roles to most of the genes
of the anthocyanin biosynthetic pathway has been achieved by genetic and biochemical
studies of maize with one notable exception, the *Bronze-2* gene. It is known that the
10   characteristic coloration of *Bronze-2 (bz2)* mutants is a consequence of the
accumulation of cyanidin-3-glucoside in the cytosol. However, in wild type (*Bz2*)
plants, anthocyanins are transported into the vacuole and become purple or red. In the
mutant (*bz2*) plants, anthocyanin is restricted to the cytoplasm where it is oxidized to a
brown ("bronze") pigment. The biochemical basis for the accumulation of
15   anthocyanins in the cytosol is not known. However, Marrs *et al.*, (1995, *supra*) have
discovered that *Bz2* encodes a glutathione *S*-transferase which is responsible for
conjugating anthocyanin with GSH. It has now been discovered in the present
invention that the plant GS-*X* pump is the entity responsible for the delivery of
glutathionated anthocyanins into the vacuole.
20   Identification of the GS-*X* pump at the molecular level has served to
confirm its wide distribution and demonstrate that these transporters constitute a
multigene family within the ABC transporter superfamily. The critical finding was that
overexpression of the human multidrug resistance-associated protein (*MRP1*) gene
(Cole *et al.*, 1992, *supra*) confers increased MgATP-dependent GS-conjugate transport
25   (Muller *et al.*, 1994 *supra*; Leier *et al.,*, 1994, J. Biol. Chem. 269:27807-27810).

Several other closely related GS-*X* pump genes have been characterized.
For example, a liver-specific GS-*X* pump (*cMOAT*), mutation of which is believed to
cause hereditary hyperbilirubinemia, has been cloned (Paulusma *et al.*, 1996,Science
271:1126-1128). The present invention establishes that YCF1 is a GS-*X* pump. In

addition, as will become apparent from a reading of the present description, two plant genes, *AtMRP1* and *AtMRP2* have been discovered in the present invention to encode homologs of MRP1, YCF1 and cMOAT.

The identification of YCF1 as a vacuolar GS-*X* pump is described in detail in the experimental details section. Similarly, the identification of two plant homologs of YCF1, AtMRP1 and AtMRP2, is also described in detail in the experimental details section. Once armed with the present invention, the skilled artisan will know how to identify and isolate genes encoding other plant GS-*X* pumps involved in sequestration of a variety of compounds in plants by following the procedures described herein.

A plant gene encoding a GS-*X* pump is isolated using any one of several known molecular procedures. For example, primers comprising conserved regions of the sequences of any of *YCF1*, *AtMRP1* or *AtMRP2*, or in fact primers comprising conserved regions of any MRP subclass (*i.e.*, probes directed to human *MRP1 cMOAT*, and other MRP genes) may be used as probes to isolate, by polymerase chain reaction (PCR) or by direct hybridization, as yet unknown *YCF1*, *AtMRP1* or *AtMRP2* homologs in a DNA library comprising specific plant DNAs. Alternatively, antibodies directed against YCF1, AtMRP1 or AtMRP2 may be used to isolate clones encoding a GS-*X* pump from an expression library comprising specific plant DNAs. The isolation of primers, probes, molecular cloning and the generation of antibodies are procedures that are well known in the art and are described, for example, in Sambrook *et al.* (1989, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor, New York) and in Harlow *et al.* (1988, Antibodies, A Laboratory Manual, Cold Spring Harbor, New York).

The invention includes an isolated DNA encoding a plant GS-*X* pump capable of transporting a glutathionated compound across a biological membrane. Preferably, the membrane is derived from a cell. Preferably, the DNA encoding a plant GS-*X* pump is at least about 40% homologous to at least one of *YCF1*, *AtMRP1* or *AtMRP2*. More preferably, the isolated DNA encoding a plant GS-*X* pump is at least

about 50%, even more preferably, at least about 60%, yet more preferably, at least

about 70%, even more preferably, at least about 80%, yet more preferably, at least ·

about 90% homologous, and more preferably, at least about 99% homologous to at

least one of *YCF1*, *AtMRP1* or *AtMRP2*. More preferably, the isolated DNA encoding

5   a plant GS-*X* pump is *Arabidopsis AtMRP1* or *AtMRP2*. Most preferably, the isolated

DNA encoding a plant GS-*X* pump is SEQ ID NOS:1, 2, 4 or 5.

Thus, the invention should be construed to include genes which encode

Arabidopsis AtMRP1 and AtMRP2 and *Arabidopsis AtMRP1* and *AtMRP2*-related

genes.

10   By "GS-*X* pump" as used herein, is meant a protein which transports a

glutathione-conjugated compound across a biological membrane.

By the term "DNA encoding a GS-*X* pump" as used herein is meant a

gene encoding a polypeptide capable of transporting a glutathionated compound across

a biological membrane.

15   By "*AtMRP*-related gene" as used herein, is meant a gene encoding a

GS-*X* pump which is a member of the *MRP/YCF1/cMOAT* family of genes. An

AtMRP1 or *AtMRP2*-related gene may be present in a cell which also encodes an

AtMRP gene or it may be present in a different cell and in a different plant species.

As described in the Experimental Detail section, *AtMRP* genes encode

20   proteins which have specific domains located therein, namely, the N-terminal

extension, transmembrane spans, TM1 and TM2, nucleotide binding folds, NBF1 and

NBF2, putative CFTR-like regulatory domain (R) and the C-terminus. An *AtMRP*-

related gene is therefore also one in which selected domains in the related protein share

significant homology (at least about 40% homology) with the same domains in either

25   of YCF1, AtMRP1 or AtMRP2. For example, when the R-domain in the AtMRP-

related protein shares at least about 40% homology with the R domain in YCF1,

AtMRP1 or AtMRP2, and when the product of that is a GS-*X* pump, then that gene is

an *AtMRP*-related gene. Similarily, when the N-terminal extension in the AtMRP-

related protein shares at least about 40% homology with the N-terminal extension in

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YCF1, AtMRP1 or AtMRP2, and when the product of that is a GS-*X* pump, then that

gene is an *AtMRP*-related gene. It will be appreciated that the definition of an *AtMRP*-

related gene encompasses those genes having at least about 40% homology in any of

the described domains contained therein with the same or a similar domain in either of

5   YCF1, AtMRP1 or AtMRP2. In addition, when the term homology is used herein to

refer to the domains of these proteins, it should be construed to be applied to homology

at both the nucleic acid and the amino acid levels.

        While a significant homology between similar domains in *AtMRP*-

related genes or their protein products is considered to be at least about 40%,

10   preferably, the homology between domains is at least about 50%, more preferably, at

least about 60%, even more preferably, at least about 70%, even more preferably, at

least about 80%, yet more preferably, at least about 90% and most preferably, the

homology between similar domains is about 99% between a domain in an *AtMRP*-

related gene or protein product thereof, and at least one of *YCF1, AtMRP1* or *AtMRP2*

15   or the protein products thereof.

        Plants from which *AtMRP1, AtMRP2* or *YCF1* related genes may be

isolated include any plant in which the GS-*X* pump is found, including, but not limited

to, soybean, castor bean, maize, petunia, potato, tomato, sugar beet, tobacco, oats,

wheat, barley, pea, faba bean and alfalfa.

20   By the term "glutathionated-conjugated compound" as used herein is

meant a compound, *e.g.,* a metal, a xenobiotic, a isoflavonoid phytoalexin, anthocyanin

or auxin, which is chemically conjugated to glutathionine. Conjugation of compounds

to glutathione occurs naturally within cells and organisms and may also be

accomplished enzymatically or non-enzymatically *in vitro* as described herein in the

25   experimental details section.

        Also included in the invention is an isolated DNA encoding a

biologically active polypeptide fragment of a plant GS-*X* pump. Preferably, the

isolated DNA encoding a biologically active polypeptide fragment of a plant GS-*X*

pump is at least about 40% homologous to a biologically active polypeptide fragment

of at least one of YCF1, AtMRP1 or AtMRP2. More preferably, the isolated DNA

encoding a biologically active polypeptide fragment of a plant GS-X pump is at least

about 50%, even more preferably, at least about 60%, yet more preferably, at least

about 70%, even more preferably, at least about 80%, yet more preferably, at least

5   about 90%, and even more preferably, at least about 99% homologous to a biologically

active polypeptide fragment of at least one of YCF1, AtMRP1 or AtMRP2. Most

preferably, the isolated DNA encoding a biologically active polypeptide fragment of a

plant GS-X pump is a biologically active polypeptide fragment of *Arabidopsis*

AtMRP1 or AtMRP2.

10   Preferably, the isolated DNA encoding a biologically active polypeptide

fragment of a plant GS-X pump is about 200 nucleotides in length. More preferably,

the isolated DNA encoding a biologically active polypeptide fragment of a plant GS-X

pump is about 400 nucleotides, even more preferably, at least about 600, yet more

preferably, at least about 800, even more preferably, at least about 1000, and more

15   preferably, at least about 1200 nucleotides in length.

The invention further includes a vector comprising a gene encoding a

plant GS-X pump and a vector comprising nucleic acid sequence encoding a

biologically active fragment thereof. The procedures for the generation of a vector

encoding a plant GS-X pump, or fragment thereof, are well known in the art once the

20   sequence of the gene is known, and are described, for example, in Sambrook *et al.*

(*supra*). Suitable vectors include, but are not limited to, disarmed *Agrobacterium*

tumor-inducing (Ti) plasmids (*e.g.*, pBIN19) containing the target gene under the

control of the cauliflower mosaic virus (CaMV) 35S promoter (Lagrimini *et al.*, 1990,

Plant Cell 2:7-18) or its endogenous promoter (Bevan, 1984, Nucl. Acids Res.

25   12:8711-8721).

Also included in the invention is a cell comprising an isolated DNA

encoding a plant GS-X pump and a cell comprising an isolated DNA encoding a

biologically active fragment thereof. Such a cell is referred to herein as a "recombinant

cell."

The procedures for the generation of a cell encoding a plant GS-X pump or fragment thereof, are well know in the art once the sequence of the gene is known, and are described, for example, in Sambrook et al. (supra). Suitable cells include, but are not limited to, yeast cells, bacterial cells, mammalian cells, and baculovirus-

5   infected insect cells transformed with the gene for the express purpose of generating GS-X polypeptide. In addition, plant cells transformed with the gene for the purpose of producing cells and regenerated plants having increased resistance to and increased capacity for heavy metal accumulation, increased resistance to organic xenobiotics and increased capacity for organic xenobiotic accumulation or altered coloration.

10   The invention also includes an isolated preparation of a polypeptide comprising a plant GS-X pump capable of transporting a glutathionated compound across a biological membrane. Preferably, the isolated preparation of a polypeptide comprising a plant GS-X pump is at least about 30% homologous to at least one of YCF1, AtMRP1 or AtMRP2. More preferably, the isolated preparation of a

15   polypeptide comprising a plant GS-X pump is at least about 40%, even more preferably, at least about 50%, yet more preferably, at least about 60%, even more preferably, at least about 70%, more preferably, at least about 80%, even more preferably, at least about 90% and more preferably, at least about 99% homologous to at least one of YCF1, AtMRP1 or AtMRP2. More preferably, the isolated preparation

20   of a polypeptide comprising a plant GS-X pump is Arabidopsis AtMRP1 or AtMRP2. Most preferably, the isolated preparation of a polypeptide comprising a plant GS-X pump is SEQ ID NOS: 3 or 6.

Also included in the invention is an isolated preparation of a biologically active polypeptide fragment of a plant GS-X pump. Preferably, the

25   isolated preparation of a biologically active polypeptide fragment of a plant GS-X pump is at least about 30% homologous to a biologically active polypeptide fragment of at least one of YCF1, AtMRP1 or AtMRP2. More preferably, the isolated preparation of a biologically active polypeptide fragment of a plant GS-X pump is at least about 40%, even more preferably, at least about 50%, yet more preferably, at least

about 60%, even more preferably, at least about 70% and yet more preferably, at least about 80%, even more preferably, at least about 90% and more preferably, at least about 99% homologous to a biologically active polypeptide fragment of at least one of YCF1, AtMRP1 or AtMRP2. Most preferably, the isolated preparation of a

5   biologically active polypeptide fragment of a plant GS-X pump is a biologically active polypeptide fragment of *Arabidopsis* AtMRP1 or AtMRP2.

Preferably, the polypeptide in the isolated preparation of a biologically active polypeptide fragment of a plant GS-X pump is about 60 amino acids in length. More preferably, the polypeptide in the isolated preparation of a biologically active

10   polypeptide fragment of a plant GS-X pump is about 130 amino acids, even more preferably, at least about 200, yet more preferably, at least about 300, even more preferably, at least about 350, and more preferably, at least about 400 amino acids in length.

As used herein, the term "homologous" refers to the subunit sequence

15   similarity between two polymeric molecules *e.g.*, between two nucleic acid molecules, *e.g.*, between two DNA molecules, or two polypeptide molecules. When a subunit position in both of the two molecules is occupied by the same monomeric subunit, *e.g.*, if a position in each of two polypeptide molecules is occupied by phenylalanine, then they are homologous at that position. The homology between two sequences is a direct

20   function of the number of matching or homologous positions, *e.g.*, if half (*e.g.*, 5 positions in a polymer 10 subunits in length) of the positions in two polypeptide sequences are homologous then the two sequences are 50% homologous; if 70% of the positions, *e.g.*, 7 out of 10, are matched or homologous, the two sequences share 70% homology. By way of example, the polypeptide sequences ACDEFG and ACDHIK

25   (SEQ ID NOS:9 and 10, respectively) share 50% homology and the nucleotide sequences CAATCG and CAAGAC share 50% homology.

An "isolated DNA," as used herein, refers to a DNA sequence which has been separated from the sequences which flank it in a naturally occurring state, *e.g.*, a DNA fragment which has been removed from the sequences which are normally

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adjacent to the fragment, *e.g.*, the sequences adjacent to the fragment in a genome in which it naturally occurs. The term also applies to nucleic acids which have been substantially purified from other components which naturally accompany the nucleic acid (*e.g.*, RNA, DNA or protein) in its natural state. The term therefore includes, for

5   example, a recombinant DNA which is incorporated into a vector; into an autonomously replicating plasmid or virus; or into the genomic DNA of a prokaryote or eukaryote; or which exists as a separate molecule (e.g., as a cDNA or a genomic or cDNA fragment produced by PCR or restriction enzyme digestion) independent of other sequences. It also includes a recombinant DNA which is part of a hybrid gene

10   encoding additional polypeptide sequence.

As used herein, the term "isolated preparation of a polypeptide" describes a polypeptide which has been separated from components which naturally accompany it. Typically, a polypeptide is isolated when at least 10%, more preferably at least 20%, more preferably at least 50%, more preferably at least 60%, even more

15   preferably at least 75%, more preferably at least 90%, and most preferably at least 99% of the total material (by volume, by wet or dry weight, or by mole per cent or mole fraction) of a sample is the polypeptide of interest. The degree of isolation of the polypeptide can be measured by any appropriate method, *e.g.*, by column chromatography, polyacrylamide gel electrophoresis, or by HPLC analysis. For

20   example, a polypeptide is isolated when it is essentially free of naturally associated components or when it is separated from the native compounds which accompany it in its natural state.

As used herein, by the term "biologically active" as it refers to GS-X pump activity as used herein, is meant a polypeptide, or a fragment thereof, which is

25   capable of transporting a glutathionated compound across a biological membrane.

In summary, the invention should be construed to include DNA comprising *AtMRP1* and *AtMRP2*, and any mutants, derivatives, homologs and fragments thereof, which encode GS-*X* pump biological activity.

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The invention further features an isolated preparation of a nucleic acid
which is antisense in orientation to a portion or all of a plant GS-*X* pump gene, wherein
the nucleic acid is capable of inhibiting expression of the GS-*X* pump gene when
introduced into cells comprising the GS-*X* pump gene. The nucleic acid is antisense to

5   either a portion or all of a plant GS-*X* pump gene, which gene is preferably *Arabidopsis
AtMRP1, Arabidopsis AtMRP2* or a homolog thereof. The "isolated preparation of a
nucleic acid" and the "portion" of the gene to which the nucleic acid is antisense,
should be of a sufficient length so as to inhibit expression of the desired target gene.
The actual length of the isolated preparation of the nucleic acid may vary, and will

10   depend on the particular target gene and the region of that gene which is targetted.
Typically, the isolated preparation of the nucleic acid will be at least about 15
contiguous nucleotides; more typically, it will be between about 15 and about 50
contiguous nucleotides, or it may even be more than 50 contiguous nucleotides in
length.

15   As used herein, a sequence of a nucleic acid is "antisense" to a portion
or all of a GS-*X* pump gene when the sequence of nucleic acid does not encode a GS-*X*
polypeptide. Rather, the sequence which is being expressed in the cells is identical to
the non-coding strand of the GS-*X* pump gene and thus, does not encode a GS-*X* pump
polypeptide.

20   "Complementary," as used herein, refers to the subunit sequence
complementarity between two nucleic acids, *e.g.*, two DNA molecules. When a
nucleotide position in both of the molecules is occupied by nucleotides normally
capable of base pairing with each other, then the nucleic acids are considered to be
complementary to each other at this position. Thus, two nucleic acids are

25   complementary to each other when a substantial number (at least 50%) of
corresponding positions in each of the molecules are occupied by nucleotides which
normally base pair with each other (*e.g.*, A:T and G:C nucleotide pairs).

In yet another aspect of the invention, there is provided an antibody
directed against a plant GS-*X* pump, preferably AtMRP1 or AtMRP2, which antibody

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is specific for the whole molecule or either the N-terminal or the C-terminal or internal portions of AtMRP1 or AtMRP2. Methods of generating such antibodies are well known in the art and are described, for example, in Harlow *et al.* (*supra*).

The present invention also provides for analogs of proteins or peptides encoded by *AtMRP1* or *AtMRP2*. Analogs can differ from naturally occurring proteins or peptides by conservative amino acid sequence differences or by modifications which do not affect sequence, or by both.

For example, conservative amino acid changes may be made, which although they alter the primary sequence of the protein or peptide, do not normally alter its function. Conservative amino acid substitutions typically include substitutions within the following groups:

glycine, alanine;

valine, isoleucine, leucine;

aspartic acid, glutamic acid;

asparagine, glutamine;

serine, threonine;

lysine, arginine;

phenylalanine, tyrosine.

Modifications (which do not normally alter primary sequence) include *in vivo*, or *in vitro* chemical derivatization of polypeptides, *e.g.*, acetylation, or carboxylation. Also included are modifications of glycosylation, *e.g.*, those made by modifying the glycosylation patterns of a polypeptide during its synthesis and processing or in further processing steps; *e.g.*, by exposing the polypeptide to enzymes which affect glycosylation, *e.g.*, mammalian glycosylating or deglycosylating enzymes. Also embraced are sequences which have phosphorylated amino acid residues, *e.g.*, phosphotyrosine, phosphoserine, or phosphothreonine.

Also included are polypeptides which have been modified using ordinary molecular biological techniques so as to improve their resistance to proteolytic degradation or to optimize solubility properties or to render them more suitable as a

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therapeutic agent. Analogs of such polypeptides include those containing residues other than naturally occurring L-amino acids, *e.g.*, D-amino acids or non-naturally occurring synthetic amino acids. The peptides of the invention are not limited to products of any of the specific exemplary processes listed herein.

5   The invention further includes a transgenic plant comprising an isolated DNA encoding a plant GS-*X* pump polypeptide or a fragment thereof, capable of transporting a glutathionated compound across a biological membrane. The transgenic plant of the invention may comprise a transgene encoding a plant GS-*X* pump polypeptide or a fragment thereof, or it may comprise a transgene encoding a yeast GS-

10   *X* pump polypeptide or a fragment thereof, which yeast transgene is expressed in the plant to yield a biologically active GS-*X* pump protein product. By way of example, there is provided herein in the experimental examples section a transgenic *Arabidopsis* plant comprising a yeast *YCF1* transgene, which when the transgene is expressed in the transgenic plant, confers upon the plant the ability to grow on media containing

15   concentrations of heavy metal (cadmium) or organic xenobiotic (CDNB) that otherwise prevent of nontransgenic plants.

The invention also includes a transgenic plant comprising an isolated DNA comprising the sequence of a plant GS-*X* pump polypeptide or a fragment thereof, which plant GS-*X* pump is capable of transporting a glutathionated compound

20   across a membrane derived from a cell, wherein the sequence of the isolated DNA is positioned in an antisense orientation with respect to the direction of transcription of the DNA.

Thus, included in the invention is a transgenic plant comprising an isolated DNA encoding a yeast YCF1 or a fragment thereof, capable of transporting a

25   glutathionated compound across a membrane derived from a cell.

In addition, the invention includes a transgenic plant comprising an isolated DNA comprising the sequence of a yeast *YCF1* gene or a fragment thereof, wherein the sequence of the isolated DNA is positioned in an antisense orientation with respect to the direction of transcription of the DNA.

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By "transgenic plant" as used herein, is meant a plant, the cells, the seeds and the progeny of which comprise a gene inserted therein, which gene has been manipulated to be inserted into the cells of the plant by recombinant DNA technology. The manipulated gene is designated as a "transgene."

5   By the term "nontransgenic but otherwise substantially homozygous wild type plant" as used herein, is meant a nontransgenic plant from which the transgenic plant was generated.

"Positioned in an antisense orientation with respect to the direction of transcription of the DNA" as used herein, means that the transcription product of the

10   DNA, the resulting mRNA, does not encode a GS-X pump. Rather, the mRNA comprises a sequence which is complementary to an mRNA which encodes a GS-X pump.

If vacuolar transport rate limits xenobiotic detoxification and if the amount of GS-X pump is rate limiting on the overall rate of vacuolar uptake, transgenic

15   plants with increased *YCF1, AtMRP1* or *AtMRP2* expression are expected to be more resistant to the toxic effects of glutathione-conjugable xenobiotics and capable of accumulating higher vacuolar conjugate levels than non-transgenic plants. The former property permits the sustained growth of transgenic plants in the presence of xenobiotic concentrations that would retard the growth of plants exhibiting normal levels of

20   transporter expression. The latter property confers on the plants the ability for hyperaccumulation of glutathionated xenobiotics.

Increased resistance to xenobiotics has application in herbicide technology and plant growth in habitats polluted with organics. Hyperaccumulation has application in the extraction of organic pollutants from contaminated ground soils.

25   The closest known similar technologies to those described herein (a) involve the isolation of mutants or the engineering of plants in which the target for xenobiotic action is no longer sensitive, (b) involve the generation of mutants with elevated cellular levels of glutathionine (GSH) or with increased glutathione-S-transferase activities, or (c) involve the application of chemical agents ("safeners") that

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elevate GSH and/or glutathione-S-transferase levles or activities. These known

technologies differ from the strategy proposed herein in three respects: (i) The utility

of mutated target gene products is limited in its application to those xenobiotics that

directly interact with the target in question. In contrast, the vacuolar GS-X pump is of

5   broad substrate specificity. (ii) Technologies based on elevated cellular GSH levels or

increased glutathione-S-transferase catalytic efficiencies are limited by the capacity of

cells to subsequently metabolize and/or sequester the conjugates generated. The

success of these latter technologies eventually depends on delivery of GSH-conjugates

into the vacuole and in turn, depends on the activity of the vacuolar GS-X pump. (iii)

10   Since the plant vacuole frequently constitutes 40-90% of total intracellular volume and

the GS-X pump mediates the uptake of xenobiotics into this compartment, the potential

for hyperaccumulation on a tissue weight basis is great. Hyperaccumulators may

therefore be used for the fixation/sequestration of toxins and their removal from soils.

None of the other known technologies have this characteristic.

15   The generation of transgenic plants comprising sense or antisense DNA

having the sequence of a GS-X pump or a fragment thereof, may be accomplished by

transformation of the plant with a plasmid encoding the desired DNA sequence.

Suitable vectors include, but are not limited to, disarmed *Agrobacterium* tumor-

inducing (Ti) plasmids containing a sense or antisense strand placed under the control

20   of the strong constitutive CaMV 35S promoter or under the control of an inducible

promoter (Lagrimini *et al.*, 1990, *supra*; van der Krol *et al.*, 1988, Gene 72:45-50).

Methods for the generation of such constructs, plant transformation and plant

regeneration are well known in the art once the sequence of the desired gene is known

and are described, for example, in Ausubel *et al.* (1993, *Current Protocols in*

25   *Molecular Biology*, Greene and Wiley, New York).

Suitable vector and plant combinations will be readily apparent to those

of skill in the art and can be found, for example, in Maliga *et al.* (1994, *Methods in*

Plant Molecular Biology: A Laboratory Manual, Cold Spring Horbor, New York).

Transformation of plants may be accomplished using the

Agrobacterium-mediated leaf disc transformation method described by Horsch *et al.*

(1988, *Leaf Disc Transformation, Plant Molecular Biology Manual A5*:**1**).

A number of procedures may be used to assess whether the transgenic

5   plant comprises the desired DNA. For example, genomic DNA obtained from the cells

of the transgenic plant may be analyzed by Southern blot hybridization or by PCR to

determine the length and orientation of any inserted, transgenic DNA present therein.

Northern blot hybridization analysis or PCR may be used to characterize mRNA

transcribed in cells of the transgenic plant. In situations where it is expected that the

10   cells of the transgenic plant express GS-*X* polypeptide or a fragment thereof, Western

blot analysis may be used to identify and characterize polypeptides so expressed using

antibody raised against the GS-*X* pump or fragments thereof. The procedures for

performing such analyses are well known in the art and are described, for example, in

Sambrook *et al. (supra)*.

15   The transgenic plants of the invention are useful for the manipulation of

xenobiotic detoxification, heavy metal detoxification, control of plant pathogens,

control of plant coloration, herbicide metabolism and phytohormone metabolism. For

example, a transgenic plant encoding an *AtMRP1* or an *AtMRP2* gene or an *AtMRP1*-

or *AtMRP2*-related gene, or a yeast *YCF1* or *YCF1*-related gene, is useful for

20   xenobiotic detoxification and heavy metal detoxification when grown on soil

containing xenobiotics or heavy metals. Such plants are capable of removing

xenobiotic toxins or heavy metals from the soil thereby generating soil which has

reduced levels of compounds that are detrimental to the overall health of the

environment.

25   Accordingly, the invention includes a method of removing xenobiotic

toxins from soil comprising generating a transgenic plant having a transgene encoding

a GS-*X* pump and planting the plant or the seeds of the plant in the soil wherein

xenobiotic toxins in the soil are sequestered within the plant during growth of the plant

in the soil.

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Similarly, the invention includes a method of removing heavy metals from soil comprising generating a transgenic plant having a transgene encoding a GS-X pump and planting the plant or the seeds of the plant in the soil wherein heavy metals in the soil are sequestered within the plant during growth of the plant in the soil.

5   When the levels of xenobiotic toxins or heavy metals in the soil have been sufficiently reduced, the transgenic plant may be removed from the soil and destroyed or discarded in an environmentally safe manner. For example, the harvested plants can be reduced in volume and/or weight by thermal, microbial, physical or chemical means to decrease handling, processing and potential subsequent land filling

10   costs (Cunningham et al., 1996, Plant Physiol. 110:715-719). In the case of valuable metals, subsequent smelting and recovery of the metal may be cost-effective (Raskin, 1996, Proc. Natl. Acad. Sci. USA 93:3164-3166).

This technique of remediating soil is more efficient, less expensive and easier than most chemical or physical methods. The estimated costs of remediation are

15   as follows: U.S. $10-100 per cubic meter of soil for removal of volatile or water soluble pollutants by in situ remediation using plants; U.S. $60-300 per cubic meter of soil for landfill or low temperature thermal treatment remediation of soil contaminated with the same compounds; and, U.S. $200-700 per cubic meter of soil for remediation of soil contaminated with materials requiring special landfilling arrangements or high

20   temperature thermal treatment (Cunningham et al., 1995, Trends Biotechnol. 13:393-397).

Preferably, the transgene in the transgenic plant of the invention is AtMRP1, AtMRP2, YCF1 or genes encoding fragments or analogs of AtMRP1, AtMRP2 or YCF1, or the transgene is a gene which is related to AtMRP1, AtMRP2,

25   YCF1.

The types of plants which are suitable for use in this method of the invention include, but are not limited to, high yield crop species for which cultivation practices have already been perfected, or engineered endemic species that thrive in the area to be remediated.

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In certain situations, it may be necessary to prevent the removal of substances such as xenobiotic toxins and heavy metals from the soil. In such situations, transgenic plants are generated comprising a transgene comprising a GS-X pump sequence which is in the antisense orientation with respect to transcription. Such

5   transgenes therefore serve to inhibit the function of a GS-X pump expressed in the plants thereby preventing removal of xenobiotics or heavy metals from the soil.

The production of plants having GS-X pump antisense sequences has application in the manipulation of plant/food coloration and in the diminution of organic xenobiotic (e.g., herbicide) or heavy metal accumulation by crop species. For

10   example, ingestion by animals or humans of low organic toxin/low heavy metal crops will likely contribute to an improvement in the overall health of animals and humans.

Accordingly, the invention includes a method of preventing the removal of xenobiotic toxins or heavy metals from soil comprising generating a transgenic plant having a transgene comprising a GS-X pump sequence which is in the antisense

15   orientation with respect to transcription and planting the plant or the seeds of the plant in the soil, wherein removal of xenobiotics and heavy metals from the soil is prevented during growth of the plant in the soil.

The antisense sequences which are useful for the generation of transgenic plants having antisense GS-X pump sequences are those which will inhibit

20   expression of a resident GS-X gene in the plant.

The types of plants which are suitable for use in this method of the invention using antisense sequences include, but are not limited to, plants for which anthocyanins contribute to flower, fruit or leaf coloration and food crops for which decreased organic xenobiotic and/or heavy metal accumulation is desirable.

25   In a similar manner to that described herein, a transgenic plant may be generated which exhibits increased accumulation and/or resistance to isoflavonoid alexins by introducing into the cells of the plant a transgene encoding a GS-X pump capable of transporting glutathionated isoflavonoid alexins into vacuoles in the plant, thereby isolating the isoflavonoid alexins from the cytoplasm of the cells of the plant.

Preferably, the transgene is *AtMRP1*, *AtMRP2*, *YCF1* or genes encoding fragments or

analogs of AtMRP1, AtMRP2 or YCF1, or the transgene is a gene which is related to

AtMRP1, *AtMRP2* or *YCF1*.

The invention thus includes a method of generating a pathogen-resistant

5   transgenic plant comprising introducing into the plant a transgene encoding a GS-X

pump capable of transporting glutathionated isoflavonoid alexins into vacuoles in the

plant.

The types of plants suitable for the introduction of the desired transgene

include, but are not limited to, plants which are leguminous plants, for example, alfalfa,

10   cashew nut, castor bean, faba bean, french bean, mung bean, pea, peanut, soybean and

walnut.

As discussed herein, it has also been discovered in the present invention

that the *Bz2* gene which encodes a glutathione-S-transferase, glutathionates

anthocyanins and possibly other compounds for transport by the GS-X pump. The

15   anthocyanin-derivatives so generated are subsequently transported across biological

membranes by the vacuolar GS-X pump. Vacuolar anthocyanins are responsible for the

red and purple hues of many plant organs (petals, leaves, stems, seeds, fruits, etc.).

Vacuolar anthocyanins are found in most flowering plants. However, they are not

solely responsible for plant coloration. Rather, plant coloration is determined by the

20   relative amounts and combinations in which these various pigments are accumulated.

Thus, it is possible to manipulate plant coloration by generating transgenic plants with

increased (sense DNA) or decreased (antisense DNA) expression of the GS-X pump.

Transgenic plants having GS-X pump sense sequences are expected to contain more

red/purple pigmentation that their nontransgenic but otherwise homozygous

25   counterparts and transgenic plants having GS-X pump antisense sequences are

expected to contain less red/purple pigmentation and possibly more brown

pigmentation that their nontransgenic but otherwise homozygous counterparts. The

generation of such types of transgenic plants may be accomplished following the

procedures described herein.

With respect to the aforementioned information regarding anthocyanins, it is important to note that accumulating evidence from studies of the MRP-subclass members from non-plant sources reveals that the group of transporters formerly referred to an GS-X pumps because of their affinity toward GS-conjugates, GSSG and

5   cysteinyl leukotrienes, do not transport GS-conjugates exclusively (Ishikawa et al., 1997, Bioscience Reports 17:189-208). Investigation of the human MRP1 protein, cMOAT and ScYCF1 establish that these proteins are capable of transporting a broad range of compounds in addition to GS-conjugates and GSSG Jedlitschky et al., 1996, Cancer Res. 56:988-994; Paulusma et al., 1996, Science 271:1126-1128; Jansen et al.,

10   1987, Hepatol. 7:71-76; Sathirakul et al., 1993, J. Pharmacol. Exp. Therap. 268:65-73). Thus, these proteins transport non-glutathionated compounds.

It has been discovered in the present invention that the plant proteins, AtMRP1 and AtMRP2, differ in their substrate preferences. For example, no only does AtMRP2 exhibit a much higher transport capacity than does AtMRP1, but AtMRP2

15   has the capacity to transport chlorophyll breakdown products in leaf senescence, which breakdown products are not glutathionated. Thus, according to the present invention, it is possible to manipulate plant coloration by changing the relative levels of expression of various members of this class of transporters in a plant cell. It is possible, using the information provided herein, to affect the rate of breakdown of chlorophyll, for

20   example, by manipulating the expression of AtMRP2 in a plant cell.

In addition to the above, there is provided as part of the invention, *AtMRP1* and *AtMRP2* promoter sequences. By operably coupling the *AtMRP1* or *AtMRP2* promoters to other genes, it may be possible to confer on these other genes expression characteristics similar to those of *AtMRP1* or *AtMRP2*, namely, modulation

25   by xenobiotics, plant pathogens, etc. The data which are presented herein include the promoter sequences of these genes, which promoter sequences are useful in a variety of applications in plants. For example, GS-X pump activity which is associated with herbicide metabolism (exemplified by organic xenobiotic transport), heavy metal sequestration (exemplified by cadmium transport), plant-pathogen interactions

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(exemplified by vacuolar uptake of medicarpin), plant cell pigmentation (exemplified by transport of glutathionated anthocyanins) and plant hormone metabolism (exemplified by the transport of glutathionated auxins) may be examined as a result of the present invention. The present invention facilitates the identification of plants and cells therein which are capable of GS-X pump activity, and further facilitates the exploitation of plant cell GS-X pump activity for the purpose of affecting plant function with respect to herbicide metabolism, heavy metal sequestration, plant-pathogen interactions, plant cell pigmentation and plant hormone metabolism.

The invention includes an isolated DNA comprising a plant GS-X pump promoter sequence capable of driving expression of a plant GS-X pump gene, which gene is capable of transporting a glutathionated compound across a biological membrane. Preferably, the membrane is derived from a cell.

Preferably, the isolated DNA comprising a plant GS-X pump promoter sequence is at least about 40% homologous to at least one of the *AtMRP1* or *AtMRP2* promoter sequences presented herein in Figures 23 and 24, respectively. More preferably, the isolated DNA comprising a plant GS-X pump promoter sequence is at least about 50%, even more preferably, at least about 60%, yet more preferably, at least about 70%, even more preferably, at least about 80%, yet more preferably, at least about 90% homologous, and more preferably, at least about 99% homologous to at least one of *AtMRP1* or *AtMRP2* promoter sequences presented herein in Figures 23 and 24, respectively. Most preferably, the isolated DNA comprising a plant GS-X pump promoter sequence is *Arabidopsis AtMRP1* or *AtMRP2* as shown in Figures 1 and 2, respectively.

Thus, the invention should be construed to include isolated DNA sequences comprising promoter sequences which in their natural form drive expression of genes which encode *Arabidopsis AtMRP1* and *AtMRP2* and *Arabidopsis AtMRP1* and *AtMRP2*-related genes. Once armed with the present invention, it is a simple matter to isolate sequences which are related to those shown in Figures 1 and 2. For example, conventional hybridization technology and/or PCR technology may be

employed, primers may be designed using the sequences provided herein, data bases

may be searched and the like. Procedures for the isolation of promoter sequences

which are related to those described herein are described in Sambrook et al. (1989,

Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, NY) and in Ausubel

5   *et al.* (1993, *Current Protocols in Molecular Biology*, Greene and Wiley, New York).

By the term "promoter sequence" as used herein, is meant a DNA

sequence which is required for expression of a gene which is operably linked thereto.

In some instances, this sequence may be a core promoter sequence and in other

instances, this sequence may also include an enhancer sequence and other regulatory

10   elements which are required for expression of the gene in a tissue-specific manner.

Thus, a promoter sequence must include an RNA polymerase binding site and may

include appropriate transcription factor binding sites as are necessary for activation of

transcription and expression of the gene to which the promoter sequence is attached at

the 5' end of the gene.

15   Typically, the promoter sequence of the invention comprises at least

about 150 bp in length. More typically, the promoter sequence comprises at least about

300 bp in length. More typically, the promoter sequence comprises at least about 400

bp, even more typically, at least about 500 bp, yet more typically, at least about 600 bp,

even more typically, at least about 800 bp, yet more typically, at least about 1000 bp

20   and even more typically, at least about 1200 or more bp in length.

The promoter sequence of the invention may also comprise discrete

sequences (elements) which function to regulate the activity of the promoter.

Frequently, such elements respond to the presence or absence of environmental factors,

thereby controlling gene expression in direct response to factors which are associated

25   with the environmental mileau of the plant. The response of the plant to these factors

affects the overall well-being of the plant. Elements which may be present in the

promoter sequence of the invention include, but are not limited to, a Myb recognition

sequence, a xenobiotic regulatory element, an antioxidant response element, a bZIP

recognition sequence, and the like.

Plants from which *AtMRP1*- or *AtMRP2*-related genes and therefore promoter sequences, may be isolated include any plant in which the GS-*X* pump is found, including, but not limited to, soybean, castor bean, maize, petunia, potato, tomato, sugar beet, tobacco, oats, wheat, barley, pea, faba bean and alfalfa.

5   The invention further includes a vector comprising a plant GS-*X* pump promoter sequence operably fused to a reporter gene and capable of driving expression of the reporter gene. The procedures for the generation of a vector comprising a plant GS-*X* pump promoter sequence are well know in the art once the sequence of the gene is known, and are described, for example, in Sambrook et al. (*supra*). Suitable vectors

10   include, but are not limited to, disarmed *Agrobacterium* tumor-inducing (Ti) plasmids (*e.g.*, pBIN19) (Lagrimini *et al.*, 1990, *Plant Cell* 2:7-18; Bevan, 1984, *Nucl. Acids Res.* 12:8711-8721).

Also included in the invention is a cell comprising a plant GS-*X* pump promoter sequence operably fused to a reporter gene. The procedures for the generation

15   of a cell encoding a plant GS-*X* pump or fragment thereof, are well know in the art once the sequence of the gene is known, and are described, for example, in Sambrook et al. (*supra*). Suitable cells include, but are not limited to, plant cells, yeast cells, bacterial cells, mammalian cells, and baculovirus-infected insect cells. In addition, plant cells transformed with the promoter/reporter gene construct, for the purpose of

20   assessing the effect of various compounds on promoter activity are also contemplated in the invention. Normal plant cells and those plant cells having increased resistance to and increased capacity for heavy metal accumulation, increased resistance to organic xenobiotics and increased capacity for organic xenobiotic accumulation or altered coloration, which cells comprise the promoter sequence of the invention operably fused

25   to a reporter gene, are all contemplated as part of the invention. When the promoter is fused to a reporter gene, the promoter is said to be operably linked to the reporter gene.

A "reporter gene" as used herein, is one which when expressed in a cell, results in the production of a detectable product in the cell. The level of expression the

product in the cell is proportional to the activity of the promoter sequence which drives expression of the reporter gene.

By describing two nucleic acid sequences as "operably linked" as used herein, is meant that a single-stranded or double-stranded nucleic acid moiety

5   comprises each of the two nucleic acid sequences and that the two sequences are arranged within the nucleic acid moiety in such a manner that at least one of the two nucleic acid sequences is able to exert a physiological effect by which it is characterized upon the other.

Suitable reporter genes include, but are not limited to, β-glucuronidase

10   (GUS) and green fluorescent protein (GFP), although any reporter gene capable of expression and detection in plant cells which are either known or heretofore unknown, may be fused to the plant GS-X promoter sequences of the invention.

The invention further includes a transgenic plant comprising an isolated DNA comprising a plant GS-X pump promoter sequence as defined herein.

15   The generation of transgenic plants comprising a plant GS-X pump promoter sequence operably fused to a reporter gene, may be accomplished by transformation of the plant with a plasmid comprising the desired DNA sequence. Suitable vectors include, but are not limited to, disarmed *Agrobacterium* tumor-inducing (Ti) plasmids (Lagrimini *et al.*, 1990, *supra*; van der Krol *et al.*, 1988, *Gene*

20   72:45-50). Methods for the generation of such constructs, plant transformation and plant regeneration are well known in the art once the sequence of the desired nucleic acid is known and are described, for example, in Ausubel et al. (1993, *Current Protocols in Molecular Biology*, Greene and Wiley, New York).

Suitable vector and plant combinations will be readily apparent to those

25   of skill in the art and can be found, for example, in Maliga *et al.* (1994, *Methods in Plant Molecular Biology: A Laboratory Manual*, Cold Spring Harbor, New York).

Transformation of plants may be accomplished using the *Agrobacterium*-mediated leaf disc transformation method described by Horsch *et al.* (1988, *Leaf Disc Transformation, Plant Molecular Biology Manual* A5:1).

A number of procedures may be used to assess whether the transgenic plant comprises the desired DNA. For example, genomic DNA obtained from the cells of the transgenic plant may be analyzed by Southern blot hybridization or by PCR to determine the length and orientation of any inserted, transgenic DNA present therein.

5   Northern blot hybridization analysis or RT-PCR may be used to characterize mRNA transcribed in cells of the transgenic plant. In situations where it is expected that the cells of the transgenic plant express GS-*X* polypeptide or a fragment thereof, Western blot analysis may be used to identify and characterize polypeptides so expressed using antibody raised against the GS-*X* pump or fragments thereof. The procedures for

10   performing such analyses are well know in the art and are described, for example, in Sambrook et al. (*supra*).

The transgenic plants of the invention are useful for the examination of xenobiotic detoxification, heavy metal detoxification, control of plant pathogens, control of plant coloration, herbicide metabolism and phytohormone metabolism. For

15   example, a transgenic plant comprising an *AtMRP1* or an *AtMRP2* promoter sequence fused to a reporter gene is useful for the examination of xenobiotic detoxification and heavy metal detoxification when grown on soil having xenobiotic toxins or heavy metals. Such plants are useful to an understanding of the mechanisms by which GS-*X* pump gene expression is activated and are therefore useful for the eventual generation

20   of plants which are capable of removing xenobiotic toxins or heavy metals from the soil thereby generating soil which has reduced levels of compounds that are detrimental to the overall health of the environment.

The types of plants which are suitable for use include, but are not limited to, high yield crop species for which cultivation practices have already been

25   perfected, or engineered endemic species that thrive in the area to be remediated. In addition plants for which anthocyanins contribute to flower or leaf coloration and food crops for which decreased organic xenobiotic and/or heavy metal accumulation is desirable are also suitable for use in the invention. Further useful plants are those in which it is desirable that they are capable of increased accumulation and/or resistance

to isoflavonoid alexins. Plants for which pathogen resistance is desired are also useful in the invention. Such plants include, but are not limited to, plants which are leguminous plants, for example, alfalfa, cashew nut, castor bean, faba bean, french bean, mung bean, pea, peanut, soybean and walnut. In addition, plants for which it is
5   desirable to manipulate plant coloration are also useful in the invention.

The promoter sequences of *Arabidopsis* GS-*X* pump genes *AtMRP1* and *AtMRP2* are shown in Figures 23 and 24, respectively. The following should be noted. bZIP transcription factor recognition elements have the sequences CACGTG or TGACG(T/C). One of these is present in the *AtMRP2* promoter sequence, but none are
10   present in the *AtMRP1* promoter sequence. Myb transcription factor recognition elements having the sequences A(a/D)(a/D)C(G/C) and AGTTAGTTA, wherein a/D = A, G or T with A being preferred, are present in the *AtMRP1* promoter sequence, but are not present in the *AtMRP2* promoter sequence. Xenobiotic regulatory elements (XREs) having the core sequence GCGTG are found in multiple copies in the
15   promoters of cytochrome P450 monooxygenase genes and glutathione *S*-transferase genes (Rushmore et al., 1993, J. Biol. Chem. 268:11475-11478). One XRE is found in the promoter sequence of *AtMRP1*. Antioxidant response elements (AREs) consist typically of two core sequences GTGACA(A/T)(A/T)GC (SEQ ID NO:11) that are binding sites for Activator Protein-1 (AP-1) transcription factor complex (Daniel, 1993,
20   CRC Crit. Rev. Biochem 25:173-207; Friling et al., 1992, Proc. Natl. Acad. Sci. USA 89:668-672). There is only one ARE in the *AtMRP1* promoter sequence shown in Figure 23. It has been proposed that GST genes containing an ARE are induced by electrophiles and conditions that generate oxidative stress (Daniel, *supra*). RNA instability determinants having the sequence ATTTA have been found in several plant
25   GSTs. These sequences, considered to target RNAs for degradation by RNases are usually found in the 3'-UTRs of genes (Takahashi et al., 1992, Proc. Natl. Acad. Sci. USA 89:56-59). Several of these sequences are found in both the *AtMRP1* and *AtMRP2* promoter sequences presented herein. However, it is not clear whether these sequences merely reflect the AT-richness of the sequences.

To assess GS-X pump gene expression in a plant cell whether the cell is contained within a plant or whether the cell is separated from the plant, a plasmid may be generated which comprises the β-glucuronidase (GUS) reporter gene fused to a plant GS-X promoter sequence. Preferably, the promoter sequence is either *AtMRP1*

5 or *AtMRP2*. The appropriate restriction fragment is subcloned into the GUS expression vector pBI101.3 (Jefferson et al., 1987, EMBO J., 6:3901-3907). After confirming the correct reading frame by sequencing, *Agrobacterium* or any other suitable vector, is transformed with the expression construct and is then used to used to transform the plant, or the cells thereof (Valvekens et al., 1988, Proc. Natl. Acad. Sci.

10 USA 85:5536-5540).

Expression of GUS may be localized histochemically by staining with 5-bromo-4-chloro-3-indoyl β-D-glucuronide (X-Gluc) (Jefferson et al., *supra*). Sections are obtained from the plant, they are incubated in X-Gluc, cleared by boiling in ethanol and are examined under the microscope. To eliminate or enumerate

15 complications arising from the transfer of GUS reaction product between cells, the distribution of GUS expression is then further examined both immunologically and biochemically. β-glucuronidase protein is assessed using standard dot-blotting and immunolocalization techniques (Harlow et al., 1988, *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, NY) using rabbit anti-β-glucuronidase serum

20 (Clontech). Direct estimates of GUS activity are be made fluorimetrically using 4-methyl-umbelliferyl glucuronide as substrate (Jefferson et al., *supra*) after dissection and extraction of explants.

GUS reporter gene analyses enable examination of plant responses to oxidative stress and pathogens as well as herbicides. In addition, GUS reporter gene

25 analyses enable tests of whether certain pigment-rich cell types also exhibit high levels of AtMRP expression.

The *AtMRP1* and *AtMRP2* promoter sequences are also useful for manipulating the expression of other genes in plants in that, transgenic plants may be generated which contain a desired plant gene operably fused to a GS-X pump promoter

sequence. The GS-X pump promoter sequence may be an *AtMRP1* or an *AtMRP2* promoter sequence or a *YCF1* promoter sequence positioned in an orientation such that the promoter sequence drives expression of the desired gene. The desired gene may be a plant or a non-plant gene. The generation of such transgenic plants confers upon

5   the plants the ability to respond to the presence of xenobiotics and other compounds which influence the promoter activity

In considering transport substrates for GS-X pumps, the status of GSSG as an endogenous GS-conjugate (of GSH with itself) and its involvement in cellular responses to active oxygen species (AOS) should not be overlooked. The sulfhydryl

10   group of GSH confers strong nucleophilicity and the facility for reacting with AOS, such as superoxide radicals (O_2^-), hydroxyl radicals (OH·) and hydrogen peroxide. GSH is found in the majority of eukaryotes but in prokaryotes (eubacteria) it appears to be restricted to the cyanobacteria and purple bacteria (Fahey and Sundquist 1991, Adv. Enzymol. Relat. Mol. Biol. 64:1-53). Since the cyanobacteria are

15   considered to be the first group of organisms capable of oxygenic photosynthesis and these and the purple bacteria probably gave rise to plant chloroplasts and mitochondria, respectively, it has been proposed that the emergence of the capacity for GSH biosynthesis was associated with the appearance of oxygenic and oxytrophic metabolism (approximately 4×10^9 years ago) to combat the attendant problem of

20   AOS production. Most, if not all, of the factors known to elicit GST induction - pathogen attack, heavy metals, certain organic xenobiotics, wounding and ethylene - promote AOS production (Inze and Montagu 1995, Current Opinion in Biotech. 6:153-158). Intriguing, therefore, is the possibility that GS-X pumps arose from the need to detoxify AOS and the products of their action.

25   The feasibility of such a scheme has yet to be investigated systematically but a number of disparate observations are at least consistent with a close connection between oxidative stress and GS-X pump function: (i) All identified MRP-subclass transporters, including AtMRP1 and AtMRP2 recognize GSSG as a substrate. Studies of GS-X pumps originated from the discovery of ATP-dependent

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GSSG efflux from erythrocytes (Srivastava and Beutler 1969, J. Biol. Chem. 244:9-16). (ii) In *S. cerevisiae*, overexpression of yAP1, a bZIP transcription factor, not only activates the YCF1 and GSH1 genes (Wemmie et al 1994, *supra*; Wu and Moye-Rowley 1994, *supra*), the latter of which encodes γ-glutamylcysteine

5   synthetase, but also a panoply of oxidoreductases (DeRisi et al 1997, Science 278:680-686). Of the 17 genes whose mRNA levels are found to be increased by more than threefold on DNA microarrays by yAP1, more than two-thirds contain canonical upstream yAP1-binding sites (TTACTAA or TGACTAA), five bear homology to aryl-alcohol oxidoreductases and four to the general class of

10   dehydrogenases/oxidoreductases (DeRisi et al 1997, *supra*). In view of the capacity of yAP1 overexpression to confer increased resistance to hydrogen peroxide, o-phenanthroline and heavy metals (Hirata et al 1994, Mol. Gen. Genet. 242:250-257), the fact that an appreciable fraction of the yAP1-regulated target genes identified against the yeast genome project database are oxidoreductases and coregulated with

15   both *YCF1* and *GSH1*, suggests that all of these genes play a protective role during oxidative stress. (iii) Two particularly harmful and early effects of AOS production are membrane lipid peroxidation. and oxidative DNA damage which yield highly toxic 4-hydroxyalkenals (Esterbauer et al 1991, Biochem. J. 208:129-140) and base propanols (Berhane et al 1994, Proc. Natl. Acad. Sci. USA 91:1480-1484),

20   respectively. Although such a,b-unsaturated aldehydes (and their GS-conjugates) have not yet been screened against the GS-*X* pumps from plant sources, they are established substrates for mammalian GSTs (Berhane et al 1994, *supra*) and their glutathionated derivatives are transported at high efficiency by mammalian GS-*X* pumps (Ishikawa 1989, J.Biol. Chem. 264:17343-17348).

25   There is therefore also included in the invention a method of alleviating oxidative stress in a plant comprising intorducing into the cells of the plant DNA encoding a GS-*X* pump.

         The invention is further described in detail by reference to the following experimental examples. These examples are provided for purposes of

illustration only, and are not intended to be limiting unless otherwise specified. Thus, the invention should in no way be construed as being limited to the following examples, but rather, should be construed to encompass any and all variations which become evident as a result of the teaching provided herein.

5   Experimental Examples

The experimental examples described herein provide procedures and results for the isolation and characterization of yeast *YCF1* and *Arabidopsis AtMRP1* and *AtMRP2* genes, gene products and various functions ascribed thereto. Further there is described data which establish that the *Bz2* gene product exerts its effects on

10   plant coloration via the GS-*X* pump.

The data which are now described establish that *YCF1* is a vacuolar glutathione *S*-conjugate pump. The data establish that YCF1 is a membrane protein which is responsible for catalyzing MgATP-dependent, uncoupler-insensitive uptake of glutathione *S*-conjugates into the vacuole of wild type *S. cerevisiae*.

15   *YCF1* encodes a protein responsible for resistance of yeast to the effects of cadmium. However, the mechanism by which resistance to Cd^{2+} is effected was not understood until the present invention. The data presented herein demonstrate that *YCF1* confers Cd^{2+} resistance to yeast by effecting transport of Cd^{2+} out of the cytosol via a *YCF1* encoded vacuolar glutathione *S*-conjugate pump. Further, since *YCF1*

20   confers resistance to Cd^{2+} through the transport of Cd.GS complexes or derivatives thereof, it is likely also capable of transporting other metal.GS-complexes. Examples of these other complexes include, but are not limited to, mercury (Hg), zinc (Zn), platinum (Pt) and arsenic (Ar). Both Hg^{2+} and Zn^{2+} form complexes with GSH which are analogous to those formed by Cd^{2+} (Li *et al.*, 1954, *J. Am. Chem. Soc.* 76:225-229;

25   Kapoor *et al.*, 1965, *Biochem. Biophys. Acta* 100:376-383; Perrin *et al.*, 1971, *Biochem. Biophys. Acta* 230:96-104). In addition, MRP1 eliminates the Pt^{2+} glutathionine complex *bis*(glutathionato)platinum from cancer cells (Ishikawa *et al.*, 1994, *J. Biol. Chem.* 269:29085-29093). Further, the MRP1 gene is overexpressed in cisplatin-resistant human leukemia HL-60 cells, which overexpression is associated

with increased resistance to arsenite (Ishikawa *et al.*, 1996, *J. Biol. Chem.* 271:14981-14988). Both Hg^{2+} and Ar^{2+} are common environmental contaminants and Zn^{2+} is an essential micronutrient.

According to the results of the present study, vacuolar membrane vesicles from wild type *S. cerevisiae* catalyze high rates of MgATP-dependent, uncoupler-insensitive *S*-conjugate transport, and the kinetics of the transporter involved are similar to those of the mammalian and plant vacuolar GS-*X* pumps. In addition, vacuole-deficient mutants of *S. cerevisiae* exhibit markedly increased sensitivity to cadmium, leading to the belief that one requirement for efficient elimination or detoxification of this metal is maintenance of a sizable vacuolar compartment.

It is known that *S. cerevisiae* yAP-1 transcription factor transcriptionally activates both the *YCF1* gene and the *GSH1* gene (Wemmie *et al.*, 1994, *J. Biol. Chem.* 269:32592-32597; Wu *et al.*, 1994, *Mol. Cell. Biol.* 14:5832-5839). Since *GSH1* encodes γ-glutamylcysteine synthetase, an enzyme critical for GSH synthesis, expression of the *YCF1* gene and fabrication of one of the precursors for transport by the GS-*X* pump are coordinately regulated.

In the first set of experiments described below, transport of the model compounds DNP-GS and bimane-GS by isolated membrane vesicles and intact cells was examined.

Yeast Strains and Plasmids

Two strains of *S. cerevisiae* were used in these studies: DTY165 (*MATα ura3-52 his6 leu2-3,-112 his3-Δ200 trp1-901 lys2-801 suc2-Δ*) and the isogenic *ycf1*Δ mutant strain, DTY167 (*MATα ura 3-52 his6 leu2-3,-112 his3-Δ200 trp 1-901 lys2-801 suc2-Δ, ycf1::hisG*). The strains were routinely grown in rich (YPD) medium, or, when transformed with plasmid containing functional *YCF1* gene, in synthetic complete medium (Sherman *et al.*, 1983, *Methods in Yeast Genetics*, Cold Spring Harbor Laboratory, New York) or AHC medium (Kim *et al.*, 1994, *supra*) lacking the appropriate amino acids. *Escherichia coli* strains XL1-blue (Stratagene)

and DH11S were employed for the construction and maintenance of plasmid stocks
(Ausubel *et al.*, 1987, *Current Protocols in Molecular Biology*, Wiley, New York).

        Plasmid pYCF1-HA, encoding epitope-tagged *YCF1*, was constructed
in several steps. A 1.4-kb *Sal*I-*Hind*III fragment, encompassing the carboxyl-terminal

5   segment of the open reading frame of *YCF1*, from pIBIYCF1 (Szczypka *et al.*, 1994,
supra), was subcloned into pBluescript KS⁻. Single-stranded DNA was prepared and
used as template to insert DNA sequence encoding the human influenza hemagglutinin
12CA5 epitope immediately before the termination codon of the *YCF1* gene by
oligonucleotide-directed mutagenesis. The sequence of the primer for this reaction,

10   with the coding sequence for the 12CA5 epitope underlined, was 5'-
GTTTCACAGTTTAA<u>AGCGTAGTCTGGGACGTCGTATGGGTA</u>ATTTTCATTG
ACC-3' (SEQ ID NO:12). After confirming the boundaries and fidelity of the HA-tag
coding region by DNA sequencing, the 1.4-kb *Sal*I-*Hind*III DNA fragment was
exchanged with the corresponding wild type segment of pJAW50 (Wemmie *et al.*,

15   1994, *supra*) to generate pYCF1-HA.

<u>Isolation of Vacuolar Membrane Vesicles</u>

        For the routine preparation of vacuolar membrane vesicles, 15 ml of
stationary phase cultures of DTY165 or DTY167 were diluted into 1-liter volumes of
fresh YPD medium, grown for 24 hours at 30°C to an $OD_{600\ nm}$ of approximately 0.8

20   and collected by centrifugation. After washing with distilled water, the cells were
converted to spheroplasts with Zymolyase 20T (ICN) (Kim *et al.*, 1994, *supra*) and
intact vacuoles were isolated by flotation centrifugation of spheroplast lysates on
Ficoll 400 step gradients as described by Roberts *et al.* (1991, *Methods. Enzymol.*
194:644-661). Both the spheroplast lysis buffer and Ficoll gradients contained 2

25   mg/ml bovine serum albumin, 1 µg/ml aprotinin, 1 µg/ml leupeptin, 1 µg/ml pepstatin,
and 1 mM PMSF to minimize proteolysis. The resulting vacuole fraction was
vesiculated in 5 mM $MgCl_2$, 25 mM KCl, 10 mM Tris-Mes (pH 6.9) containing 2
mg/ml bovine serum albumin, 1 µg/ml aprotinin, 1 µg/ml leupeptin, 1 µg/ml pepstatin,
and 1 mM PMSF, pelleted by centrifugation at 37,000 x g for 25 min, and resuspended

in suspension medium (1.1 M glycerol, 2 mM dithiothreitol, 1 mM Tris-EGTA, 2 mg/ml bovine serum albumin, 1 µg/ml aprotinin, 1 µg/ml leupeptin, 1 µg/ml pepstatin, 1 mM PMSF, 5 mM Tris-Mes, pH 7.6) (Kim *et al.*, 1995, *J. Biol. Chem.* 270:2630-2635).

5   In experiments involving cadmium transport, dithiothreitol and EGTA were removed from the suspension medium to prevent the attenuation of YCF1-dependent Cd^{2+} transport otherwise exerted by these compounds. Vesiculated vacuolar membranes were subjected to three cycles of 50-fold dilution into simplified suspension medium (1.1 M glycerol, 5 mM Tris-Mes, pH 8.0), centrifugation at

10   100,000 x g for 35 minutes and resuspension in the same medium before use.

For the experiment shown in Figure 4, 1 ml of partially purified vacuolar membrane vesicles (1.1-1.2 mg of protein), prepared by Ficoll flotation, were subjected to further fractionation by centrifugation through a 30-ml linear 10-40% (w/v) sucrose density gradient at 100,000 x g for 2 hours. Successive fractions were

15   collected from the top of the centrifuge tube and, after determining sucrose concentration refractometrically, the fractions were diluted with suspension medium. The diluted fractions were sedimented at 100,000 x g and resuspended in 100-µl aliquots of suspension medium for assay. For the immunoblots shown in Figure 5 and the marker enzyme analyses shown in Table 4, crude microsomes were prepared by

20   homogenization of spheroplasts in suspension medium and the sedimentation of total membranes at 100,000 x g for 35 minutes.

Microsomes and purified vacuolar membranes that were to be employed for SDS-polyacrylamide gel electrophoresis and immunoblotting were washed free of bovine serum albumin by three rounds of suspension in suspension

25   medium minus bovine serum albumin and centrifugation at 100,000 x g for 35 minutes. The final membrane preparations were either used immediately or frozen in liquid nitrogen and stored at -85°C.

Measurement of Marker Enzyme Activities

α-Mannosidase was determined according to Opheim (1978, *Biochem. Biophys. Acta* 524:121-125) using *p*-nitrophenyl-α-D-mannopyranoside as substrate. NADPH-cytochrome *c* reductase was estimated as FMN-promoted reduction of NADPH (Kubota *et al.*, 1977, *J. Biol. Chem.* 81:197-201). GDPase was measured as

5   the rate of liberation of P_i from GDP (Yanagisawa *et al.*, 1990, *J. Biol. Chem.* 265:19351-19355) in reaction buffer containing 0.05% (w/v) Triton X-100. V-ATPase, F-ATPase, and P-ATPase were assayed as bafilomycin A_1 (1 μM), azide (1 mM), and vanadate (100 μM) inhibited ATPase activity, respectively, at pH 8.0 (V-ATPase, F-ATPase) or pH 6.5 (P-ATPase) (Rea and Turner, 1990, *Methods Plant*

10   *Biochem.* 3:385-405).

Measurement of DNP-GS Uptake

Unless otherwise indicated, [^{3}H]DNP-GS uptake was measured at 25 °C in 200 μl reaction volumes containing 3 mM ATP, 3 mM $MgSO_4$, 5 μM gramicidin-D, 10 mM creatine phosphate, 16 units/ml creatine kinase, 50 mM KCl, 1 mg/ml

15   bovine serum albumin, 400 mM sorbitol, 25 mM Tris-Mes (pH 8.0), and 66.2 μM [^{3}H]DNP-GS (8.7 mCi/mmol) (Li *et al.*, 1995, *supra*). Gramicidin D was included in the uptake medium to abolish the H^+ electrochemical potential difference ($\Delta\mu_H+$) that would otherwise be established by the V-ATPase in medium containing MgATP. Uptake was initiated by the addition of vacuolar membrane vesicles (10-15 μg of

20   membrane protein), brief mixing of the samples on a vortex mixer and uptake was then allowed to proceed for 1-60 minutes. Uptake was terminated by the addition of 1 ml of ice-cold wash medium (400 mM sorbitol, 3 mM Tris-Mes, pH 8.0) and vacuum filtration of the suspension through prewetted Millipore HA cellulose nitrate membrane filters (pore diameter, 0.45 μm). The filters were rinsed twice with 1 ml of

25   ice-cold wash medium and air-dried, and radioactivity was determined by liquid scintillation counting in BCS mixture (Amersham Corp.). Nonenergized [^{3}H]DNP-GS uptake and extravesicular solution trapped on the filters were enumerated by the same procedure except that ATP and Mg^{2+} were omitted from the uptake medium.

Fluorescence Microscopy

Cells were grown in YPD medium for 24 hours at 30°C to an $OD_{600 nm}$

of approximately 1.4, and 100 μl aliquots of the suspensions were transferred to 15 ml

volumes of fresh YPD medium containing 100 μM *syn*-($ClCH_2,CH_3$)-1,5-

diazabicyclo-[3.3.0]-octa-3,6-dione-2,8-dione (monochlorobimane) (Kosower *et al.*,

5   1980, *J. Am. Chem. Soc.* **102**:4983-4993). After incubation for 6 hours, the cells were

pelleted by centrifugation, washed twice with YPD medium lacking

monochlorobimane, and viewed without fixation under an Olympus BH-2

fluorescence microscope equipped with a BP-490 UV excitation filter, AFC-0515

barrier filter, and Nomarski optics attachment.

10   Electrophoresis and Immunoblotting

Membrane samples were subjected to one-dimensional SDS-

polyacrylamide gel electrophoresis on 7-12% (w/v) concave exponential gradient gels

after delipidation with acetone:ethanol (Parry *et al.*, 1989, *J. Biol. Chem.* **264**:20025-

20032). The separated polypeptides were electrotransferred to 0.45 μm nitrocellulose

15   filters at 60 V for 4 hours at 4°C in a Mini Trans-Blot transfer cell (Bio-Rad) and

reversibly stained with Ponceau-S (Rea *et al.*, 1992, *Plant Physiol.* **100**:723-732). The

filters were blocked and incubated overnight with mouse anti-HA monoclonal

antibody (20 μg/ml) (Boehringer-Mannheim). Immunoreactive bands were visualized

by reaction with horseradish peroxidase-conjugated goat anti-mouse IgG (1/1000

20   dilution) (Boehringer-Mannheim) and incubation in buffer containing H_2O_2 (0.03%

w/v), diaminobenzidine (0.6 mg/ml) and $NiCl_2$ (0.03% w/v) (Rea *et al.*, 1992, *supra*).

Purification of Cadmium-Glutathione Complexes

Singly radiolabeled $^{109}Cd.GS_n$ and doubly radio-labeled

$^{109}Cd[^3H].GS_n$ complexes were prepared by sequential gel-filtration and anion-

25   exchange chromatography of the reaction products generated by incubating 20 mM

$^{109}CdSO_4$ (78.4 mCi/mmol) with 40 mM GSH or 40 mM [^3H]GSH (240 mCi/mmol)

in 15 ml 10 mM phosphate buffer (pH 8.0) containing 150 mM KNO_3 at 45°C for 24

hours. For gel-filtration, 2 ml aliquots of the reaction mixture were applied to a

column (40 x 1.5 cm ID) packed with water-equilibrated Sephadex G-15, eluted with

deionized water and ^{109}Cd and/or ^{3}H in the fractions was measured by liquid

scintillation counting. The fractions encompassed by each of the two ^{109}Cd.GS$_n$ peaks

identified were pooled, lyophilized and redissolved in 4 ml of loading buffer (5 mM

Tris-Mes, pH 8.0). For anion-exchange chromatography, 0.5 ml aliquots of the

5 resuspended lyophilizates from gel-filtration chromatography were applied to a Mono-

Q HR5/5 column (Pharmacia) equilibrated with the same buffer. Elution was with a

linear gradient of NaCl (0.5 ml/minute; 0-500 mM) dissolved in loading buffer. The

individual fractions corresponding to the major peaks of ^{109}Cd obtained from the

Mono-Q column (one each for the peaks resolved by gel-filtration chromatography)

10 were pooled, lyophilized and resuspended in 4 ml deionized water after liquid

scintillation counting. Buffer salts were removed before transport measurements or

mass spectrometry by passing the samples down a column (120 x 1.0 cm ID) packed

with water-equilibrated Sephadex G-15.

Measurement of ^{109}Cd^{2+} Uptake

15 MgATP-energized, uncoupler-insensitive ^{109}Cd^{2+} uptake by vacuolar

membrane vesicles was measured at 25°C in 200 μl reaction volumes containing 3

mM ATP, 3 mM MgSO$_4$, 5 μM gramicidin-D, 10 mM creatine phosphate, 16 units/ml

creatine kinase, 50 mM KCl, 400 mM sorbitol, 25 mM Tris-Mes (pH 8.0) and the

indicated concentrations of ^{109}CdSO$_4$, GSH or ^{109}Cd- and/or ^{3}H-labeled purified

20 Cd.GS$_n$ complexes as described herein except that the wash media contained 100 μM

CdSO$_4$ in addition to sorbitol (400 mM) and Tris-Mes (3 mM, pH 8.0).

Pretreatment of DTY165 Cells with Cd^{2+} or 1-Chloro-2,4-dinitrobenzene

For studies on the inducibility of *YCF1* expression and YCF1-

25 dependent transport, DTY165 cells were grown in YPD medium (Sherman *et al.*,

1983, *supra*) for 24 hours at 30°C to an OD$_{600 nm}$ of 1.0-1.2, pelleted by

centrifugation and resuspended in fresh YPD medium containing CdSO$_4$ (200 μM) or

1-chloro-2,4-dinitrobenzene (CDNB). After washing in distilled water, total RNA was

extracted and vacuolar membrane vesicles were prepared from the pretreated cells.

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Control RNA and membrane samples were prepared from DTY165 cells treated in an identical manner except that CdSO$_4$ and CDNB were omitted from the second incubation cycle.

RNase Protection Assays

5   Cd^{2+} and CDNB-elicited increases in *YCF1* mRNA levels were assayed by RNase protection using 18S rRNA as an internal control. *YCF1*-specific probe was generated by PCR amplification of the full-length *YCF1::HA* gene, encoding human influenza hemagglutinin 12CA5 (HA) epitope-tagged *YCF1*, using plasmid pYCF1-HA as template. The forward *YCF1*-specific primer and backward primer containing

10   the HA-tag coding sequence had the sequences 5'-AAACTGCAGATGGCTGGTAATCTTGTTTC-3' (SEQ ID NO:13) and 5'-GCCTCTAGATCAAGCGTAGTCTGGGACGTCGTATGGGTAATTTTCATTGA-3' (SEQ ID NO:14), respectively. An 18S rRNA-specific probe was synthesized by PCR of *S. cerevisiae* genomic DNA using sense and antisense primers having the sequences

15   5'-AGATTAAGCCATGCATGTCT-3' (SEQ ID NO:15) and 5'-TGCTGGTACCAGACTTGCCCTCC-3' (SEQ ID NO:16), respectively. Both PCR products were individually subcloned into pCR™II vector (Invitrogen) to generate plasmids pCR-*YCF1* and pCR-Y18S. After linearization of pCR-*YCF1* and pCR-Y18S with *Afl*I and *Nco*I, a 320-nucleotide *YCF1*-specific RNA probe and 220-

20   nucleotide 18S rRNA-specific probe were synthesized using T7 RNA polymerase and SP6 RNA polymerase, respectively. Aliquots of total RNA, prepared as described (Kohrer *et al.*, 1991, *Methods in Enzymol.* 194:390-398)), from control, CdSO$_4$- or CDNB-pretreated DTY165 cells were hybridized with a mixture of ^{32}P-labeled *YCF1* antisense probe (1 x 10^6 cpm) and 18S rRNA antisense probe (5 x 10^2 cpm) and

25   RNase protection (Teeter *et al.*, 1990, *Mol. Cell. Biol.* 10:5728-5735) was assayed using an RPAII kit (Ambion).

Matrix-Assisted Laser Desorption Mass Spectrometry (MALD-MS)

The ^{109}Cd.GS$_n$ complexes purified by gel-filtration and anion-exchange chromatography were adjusted to a final concentration of 2-5 mM (as Cd)

with deionized water, mixed with an equal volume of sinapinic acid (10 mg/ml)
dissolved in acetonitrile/H$_2$0/trifluoroacetic acid (70:30:0.1 % (v/v)) and applied to the
ion source of a PerSeptive Biosystems Voyager RP Biospectrometry Workstation.
The instrument, which was equipped with a 1.3 m flight tube and variable two-stage

5   ion source set at 30 kV, was operated in linear mode. Mass/charge (m/z) ratio was
measured by time-of-flight after calibration with external standards.

Protein Estimations

Protein was estimated by a modification of the method of Peterson
(1977, *Anal. Biochem.* **83**:346-356).

10   Chemicals

S-(2,4-dinitrophenyl)glutathione (DNP-GS) was synthesized from 1-
chloro-2,4-dinitrobenzene (CDNB) and GSH by the procedure of Kunst *et al.* (1983,
Biochem. Biophys. Acta **983**:123-125) and (Li *et al.*, 1995, *supra*). [^{3}H]DNP-GS
(specific activity, 8.7 mCi/mmol) and bimane-GS were synthesized enzymatically and

15   purified by a modification of the procedure of Kunst *et al.* (1983, *supra*) according to
Li *et al.* (1995, *supra*). Metolachlor-GS was synthesized by general base catalysis and
purified by reverse-phase high performance liquid chromatography (Li *et al.*, 1995,
supra).

GSH and CDNB were purchased from Fluka; AMP-PNP, aprotinin,

20   ATP, creatine kinase (type I from rabbit muscle, 150-250 units/mg of protein), creatine
phosphate, FCCP, oxidized glutathione (GSSG), S-methylglutathione,
cysteinylglycine, cysteine, glutamate and gramicidin D, leupeptin, PMSF, verapamil,
and vinblastine were from Sigma; monochlorobimane was from Molecular Probes;
cellulose nitrate membranes (0.45-μm pore size, HA filters) were from Millipore;

25   [^{3}H]glutathione[(glycine-2-^{3}H]-L-Glu-Cys-Gly; 44 Ci/mmol) was from DuPont NEN;
and ^{109}CdSO$_4$ (78.44 Ci/mmol) was from Amersham Corp. Metolachlor was a gift
from CIBA-Geigy, Greensboro, NC. All other reagents were of analytical grade and
purchased from Fisher, Fluka, or Sigma.

Sensitivity to CDNB

If the *YCF1* gene product were to participate in the detoxification of *S*-conjugable xenobiotics, mutants deleted for this gene would be expected to be more sensitive to the toxic effects of these compounds than wild type cells. This is what was found (Figure 1).

The isogenic wild type strain DTY165 and the *ycf1*Δ mutant strain, DTY167, were indistinguishable during growth in YPD medium lacking CDNB; both strains grew at the same rate after a brief lag. However, the addition of CDNB to the culture medium caused a greater retardation of the growth of DTY167 cells (Figure 1B) than DTY165 cells (Figure 1A). Inhibitory concentrations of CDNB resulted in a slower, more linear, growth rate for at least 24 hours for both strains, but DTY167 underwent growth retardation at lower concentrations than did DTY165. The optical densities of the DTY167 cultures were diminished by 65, 82, 85, and 91% by 40, 50, 60, and 70 μM CDNB, respectively, after 24 hours of incubation (Figure 1B), whereas the corresponding diminutions for the DTY165 cultures were 14, 31, 59, and 92% (Figure 1A). The increase in sensitivity to CDNB conferred by deletion of the YCF1 gene was similar to that seen with cadmium.

Impaired Vacuolar DNP-GS Transport

Vacuolar membrane vesicles purified from DTY165 cells exhibited high rates of MgATP-dependent [^{3}H]DNP-GS uptake (Figure 2). Providing that creatine phosphate and creatine kinase were included in the uptake media to ensure ATP regeneration, addition of 3 mM MgATP increased the initial rate of DNP-GS uptake by 122-fold to a value of 12.2 nmol/mg/minute. The same membrane fraction from DTY167 cells, although capable of similar rates of MgATP-independent DNP-GS uptake, was only 17-fold stimulated by MgATP and capable of an initial rate of uptake of only 1.7 nmol/mg/minute (Figure 2).

Selective Impairment of Uncoupler-Insensitive Transport

Direct comparisons between vacuolar membrane vesicles from DTY165 and DTY167 cells demonstrated that deletion of the *YCF1* gene selectively abolished MgATP-energized, Δμ$_{H}$+-independent DNP-GS transport.

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Agents that dissipate both the pH (ΔpH) and electrical ($\Delta\psi$) components of the $\Delta\mu_H+$ established by the V-ATPase (FCCP, gramicidin D) or directly inhibit the V-ATPase, itself (bafilomycin A_1), decreased MgATP-dependent DNP-GS uptake by vacuolar membrane vesicles from DTY165 cells from 77.7 ± 1.0

5   nmol/mg/10 minutes to between 43.2 ± 1.0 and 47.4 ± 1.7 nmol/mg/10 minutes (Table 1). Ammonium chloride, which abolishes ΔpH while leaving $\Delta\psi$ unaffected, on the other hand, did not inhibit DNP-GS uptake (Table 1). On the basis of these characteristics, the inability of uncouplers to markedly increase the inhibitions caused by V-ATPase inhibitors, alone, and the resistance of 50-60% of total uptake to

10   inhibition by any one of these compounds (Table 1), DNP-GS uptake by vacuolar membranes from wild type cells is concluded to proceed via two parallel mechanisms: a V-ATPase inhibitor- and uncoupler-insensitive pathway that is directly energized by MgATP, and a $\Delta\mu_H+$-dependent, V-ATPase inhibitor-sensitive and uncoupler-sensitive pathway that is primarily driven by the inside-positive $\Delta\psi$ established by the

15   V-ATPase.

Of these two pathways, the $\Delta\psi$-dependent pathway predominated in membranes from DTY167 cells (Table 1). FCCP, gramicidin D, and bafilomycin A_1 diminished net DNP-GS uptake by DTY167 vacuolar membranes from 15.4 ± 0.4 nmol/mg/10 minutes to between 4.3 ± 0.3 and 6.4 ± 0.3 nmol/mg/10 minutes.

20   Moreover, although the effects of FCCP or gramicidin D and V-ATPase inhibitors in combination were slightly greater than those seen when these agents were added individually, the transport remaining was only about 10% of that seen with wild type membranes and only 2–4-fold stimulated by MgATP. In conjunction with the negligible inhibitions seen with NH_4Cl, alone, indicating that $\Delta\psi$, not ΔpH, is the

25   principal driving force for the transport activity remaining in their vacuolar membranes, DTY167 cells are inferred to be preferentially impaired in MgATP-energized, $\Delta\mu_H+$-independent DNP-GS transport.

The nonhydrolyzable ATP analog, AMP-PNP, did not promote DNP-GS uptake by vacuolar membrane vesicles from either DTY165 or DTY167 cells

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(Table 1), indicating a requirement for hydrolysis of the γ-phosphate of ATP

regardless of whether uptake was via the YCF1- or Δψ-dependent pathway.

Table 1. Effects of MgATP, MgAMP-PNP, protonophores, ionophores and V-ATPase inhibitors on [³H]DNP-GS uptake by vacuolar membrane vesicles purified from DTY165 and DTY167 cells.
Uptake was measured for 10 minutes in standard uptake medium described herein containing 66.2 µM [³H]DNP-GS plus the compounds indicated. MgATP (3 mM) was present throughout unless otherwise indicated. MgAMP-PNP, bafilomycin A₁, FCCP, gramicidin D, and NH₄Cl were added at concentrations of 3 mM, 0.5 µM, 5 µM, 5 µM and 1 mM, respectively. Values outside parentheses are means ± SE (n = 3-6); values inside parentheses are rates of uptake expressed as percentage of control.

ADDITIONS	DNP-GS UPTAKE	
	DTY165	DTY167
	(nmol/mg/10 minutes)	
Control	77.7 ± 1.0 (100)	15.4 ± 0.4 (100)
-MgATP	2.2 ± 0.4 (2.8)	1.5 ± 0.6 (9.7)
MgAMP-PNP(-MgATP)	2.5 ± 0.5 (3.2)	1.4 ± 0.3 (9.1)
FCCP	47.4 ± 1.7 (61.0)	6.4 ± 0.3 (41.8)
Gramicidin D	45.8 ± 1.4 (58.9)	5.8 ± 0.1 (37.7)
NH₄Cl	69.1 ± 2.9 (88.9)	14.9 ± 0.7 (96.8)
NH₄Cl + gramicidin D	42.6 ± 1.8 (54.8)	4.1 ± 0.2 (26.6)
Bafilomycin A₁	43.2 ± 1.0 (55.6)	4.3 ± 0.3 (27.9)
Bafilomycin A₁ + gramicidin D	39.2 ± 2.6 (50.5)	3.8 ± 0.1 (24.7)

Abolition of High Affinity, Uncoupler-insensitive Uptake

Examination of the concentration dependence of [³H]DNP-GS uptake

revealed a near total abolition of high affinity, MgATP-dependent, uncoupler-

insensitive transport by vacuolar membrane vesicles from the *ycf1*Δ mutant strain

5   (Figure 3). When measured in the presence of uncoupler (gramicidin D), the rate of

DNP-GS uptake by vacuolar membrane vesicles purified from DTY165 cells increased

as a simple hyperbolic function of MgATP (Figure 3A) and DNP-GS concentration

(Figure 3B) to yield K_m values OF 86.5 ± 29.5 µM (MgATP) and 14.1 ± 7.4 µM

(DNP-GS) and a V_{max} of 51.0 ± 6.3 nmol/mg/10 minutes (DNP-GS). By contrast, uncoupler-insensitive uptake by the corresponding membrane fraction from DTY167 cells was more than 15-fold slower over the entire concentration range, showed no evidence of saturation and increased as a linear function of both DNP-GS and MgATP

5   concentration (Figure 3).

<u>Selective Inhibitors of YCF1-mediated Transport</u>

MgATP-dependent, uncoupler-insensitive DNP-GS uptake by vacuolar membrane vesicles purified from DTY165 cells was sensitive to inhibition by vanadate, vinblastine, verapamil, GSSG and glutathione S-conjugates other than DNP-

10   GS (Tables 2 and 3). One hundred μM concentrations of metolachlor-GS, azidophenacyl-GS and bimane-GS and 1 mM GSSG inhibited uptake by about 50% (Table 2), while vanadate, vinblastine, and verapamil exerted 50% inhibitions at concentrations of 179, 89 and 203 μM, respectively (Table 3). None of these agents significantly inhibited residual MgATP-dependent, uncoupler-insensitive DNP-GS

15   uptake by vacuolar membrane vesicles from DTY167 cells (Tables 2 and 3).

TABLE 2. Effects of GSH, GSSG, and glutathione S-conjugates other than DNP-GS on MgATP-dependent, uncoupler-insensitive [^{3}H]DNP-GS uptake by vacuolar membrane vesicles purified from DTY165 and DTY167 cells. Uptake was measured as described for Table 1 except that 5 µM gramicidin D was included in all of the uptake media. Values outside parentheses are means ± SE (n = 3-6); values inside parentheses are rates of uptake expressed as percentage of control.

COMPOUND	DNP-GS UPTAKE	
	DTY165	DTY167
	(nmol/mg/10 minutes)	
Control	47.9 ± 2.5 (100)	6.5 ± 0.8 (100)
GSH (1 mM)	50.6 ± 2.3 (105.6)	4.6 ± 1.1 (70.8)
GSSG (1 mM)	26.0 ± 0.9 (54.3)	4.4 ± 0.4 (67.7)
Metolachlor-GS (100 µM)	27.6 ± 0.9 (57.7)	4.8 ± 0.7 (73.8)
Azidophenacyl-GS (100 µM)	16.0 ± 1.4 (33.5)	5.2 ± 0.3 (80.0)
Bimane-GS (100 µM)	25.2 ± 1.1 (52.6)	4.5 ± 0.4 (69.2)

TABLE 3. Sensitivity of MgATP-dependent, uncoupler-insensitive [^{3}H]DNP-GS uptake by vacuolar membrane vesicles purified from DTY165 and DTY167 cells to inhibition by vanadate, vinblastine, and verapamil. Uptake was measured as described in Table 1 except that 5 µM gramicidin D was included in all of the uptake media. The concentrations of the compounds causing 50% inhibition of uptake (I_{50} values) were estimated by nonlinear least squares analysis after fitting the data to a single negative exponential (Marquardt, 1963, *supra*).

Addition	I_{50}	
	DTY165	DTY167
	µM	
Vanadate	179.1	Insensitive
Vinblastine	88.8	> 500
Verapamil	202.6	Insensitive

Vacuolar Membrane Localization

The capacity for MgATP-dependent, uncoupler-insensitive [^{3}H]DNP-GS uptake strictly copurified with the vacuolar membrane fraction (Table 4). By

comparison with crude microsomes (total membranes) prepared from whole
spheroplast homogenates of DTY165 cells, vacuolar membrane vesicles derived from
vacuoles purified by the Ficoll flotation technique were coordinately enriched for DNP-
GS uptake and for both of the vacuolar membrane markers assayed, α-mannosidase and

5   bafilomycin A_1-sensitive ATPase (V-ATPase) activity. The respective enrichments of
MgATP-dependent, uncoupler-insensitive DNP-GS uptake, α-mannosidase and
bafilomycin A_1-sensitive ATPase activity were 28-, 53- and 22-fold. By contrast, the
vacuolar membrane fraction was 4.5-, 6.3-, 11.1- and 4.3-fold depleted of NADPH
cytochrome c reductase (endoplasmic reticulum), latent GDPase (Golgi), vanadate-

10   sensitive ATPase (plasma membrane), and azide-sensitive ATPase activity
(mitochondrial inner membrane), respectively. Accordingly, when vacuolar membrane
vesicles derived from Ficoll-flotated vacuoles were subjected to further fractionation on
linear 10–40% (w/v) sucrose density gradients, MgATP-dependent, uncoupler-
insensitive [^{3}H]DNP-GS uptake, α-mannosidase and bafilomycin A_1-sensitive ATPase

15   activity were found to comigrate and exhibit identical density profiles (Figure 4).

TABLE 4. Comparison of rates of MgATP-dependent, uncoupler-insensitive [^{3}H]DNP-GS transport and specific activities of marker enzymes in crude microsomes and vacuolar membrane vesicles prepared from DTY165 cells. Microsomes and vacuolar membrane vesicles were prepared from spheroplasts and the marker enzymes were assayed as described herein. Values shown are means ± SE ($n = 3$).

PREPARATION	ACTIVITY		
	DNP-GS UPTAKE	α-mannosidase	NADPH-cyt c reductase
	nmol/mg/10 min	nmol/mg/min	nmol/mg/min
Microsomes	2.5 ± 0.3	6.3 ± 0.3	88.0 ± 1.3
Vacuolar membrane	69.9 ± 1.0	329.3 ± 3.2	19.3 ± 0.6
Enrichment (-fold)	27.96	52.27	0.22

PREPARATION	ACTIVITY			
	V-ATPase	GDPase	P-ATPase	F-ATPase
	μmol/mg/h		μmol/mg/h	
Microsomes	11.7 ± 6.3	35.0 ± 1.1	37.1 ± 4.6	155.6 ± 3.0
Vacuolar membrane	253.1 ± 15.8	5.5 ± 0.1	3.2 ± 1.6	35.1 ± 8.4
Enrichment (-fold)	21.63	0.16	0.09	0.23

<u>Plasmid-encoded YCF1 Mediates Vacuolar DNP-GS Transport and CDNB Resistance</u>

Immunoblots of vacuolar membranes from pYCF1-HA-transformed DTY165 or DTY167 cells, probed with mouse anti-HA monoclonal antibody, demonstrated incorporation of YCF1-HA polypeptide into the vacuolar membrane fraction (Figure 5B). Immunoreaction with the 12CA5 epitope was not detectable in lanes loaded with membranes from pRS424-transformed cells but the same quantities of membranes prepared from pYCF1-HA-transformed cells yielded a single intensely immunoreactive band with an electrophoretic mobility (M_r = 156,200) commensurate

with a computed mass of 172 kDa for the fusion protein encoded by YCF1-HA (Figure 5B).

Direct participation of the plasmid-borne YCF1-HA gene product in DNP-GS transport and CDNB detoxification was verified by the finding that vacuolar membrane vesicles purified from pYCF1-HA-transformed DTY167 cells exhibited a 6-fold enhancement of MgATP-dependent, uncoupler-insensitive [^{3}H]DNP-GS uptake (Figure 5A) which was accompanied by a decrease in the susceptibility of such transformants to growth retardation by exogenous CDNB (Figure 6). Whereas pYCF1-HA-transformed DTY167 cells exhibited a similar resistance to growth retardation by CDNB as untransformed DTY165 cells (compare Figure 6B with Figure 1A), the same mutant strain showed neither increased vacuolar DNP-GS transport *in vitro* nor decreased susceptibility to CDNB *in vivo* after transformation with parental plasmid pRS424, lacking the YCF1-HA insert (Figure 6B).

Vacuolar Accumulation of Bimane-GS *In Vivo*

Monochlorobimane, a membrane-permeant, nonfluorescent compound, is specifically conjugated with GSH by cytosolic glutathione *S*-transferases (GSTs) to generate the intensely fluorescent, membrane-impermeant *S*-conjugate, bimane-GS (Shrieve *et al.*, 1988, *J. Biol. Chem.* 263:14107-12114; Oude Elferink *et al.*, 1993, *Hepatology* 17:343-444; Ishikawa *et al.*, 1994, *J. Biol. Chem.* 269:29085-29093). The GS-*X* pumps of both animal and plant cells exhibit activity toward a broad range of *S*-conjugates, including bimane-GS (Ishikawa *et al.*, 1994, *supra*; Martinoia *et al.*, 1993, *supra*), and DNP-GS uptake by the yeast enzyme is shown herein to be reversibly inhibited by this compound (Table 2). These data suggest competition between bimane-GS and DNP-GS for a common uptake mechanism. Exogenous monochlorobimane therefore satisfies the minimum requirements of a sensitive probe for monitoring the intracellular transport and localization of its *S*-conjugate.

Fluorescence microscopy of DTY165 and DTY167 cells after incubation in growth medium containing monochlorobimane provides direct evidence that YCF1 contributes to the vacuolar accumulation of its glutathione *S*-conjugate by

intact cells (Figure 7). DTY165 cells exhibited an intense punctate fluorescence, corresponding to the vacuole as determined by Nomarski microscopy, after 6 hours of incubation with monochlorobimane (Figures 7A and 7C). The fluorescence associated with vacuolar bimane-GS was by comparison severely attenuated in most, and

5   completely absent from many, DTY167 cells (Figures 7B and 7D).

<u>ycf1Δ Mutants are Defective in GSH-Dependent Cd^{2+} Transport</u>

Physiological (1 mM) concentrations of GSH (Kang, 1992, *Drug Metabolism and Disposition* 20:714-718) promoted Cd^{2+} uptake by vacuolar membrane vesicles purified from the wild type strain DTY165 but not the *ycf1Δ* mutant

10   strain DTY167 (Figure 8). Addition of Cd^{2+} (80 μM) to GSH-containing media elicited MgATP-dependent, uncoupler-insensitive ^{109}Cd^{2+} uptake rates of 4.5 and 0.8 nmol/mg/minute by DTY165 and DTY167 membranes, respectively (Figures 8A and 8B). Uptake by DTY165 membranes was diminished more than 9-fold by the omission of GSH (Figure 8A) whereas uptake by DTY167 membranes was slightly stimulated

15   (Figure 8B).

GSH maximally stimulated uptake within minutes ($t_{1/2}$ < 5 minutes) of the addition of Cd^{2+} to the uptake medium and uptake was sigmoidally dependent on Cd^{2+} concentration, achieving half-maximal velocity at 120 μM (Figure 8C).

<u>Specific Requirement for GSH</u>

20   The stimulatory action of GSH was abolished by the omission of MgATP from the assay medium (Figure 8 and Table 5) and 1 mM concentrations of GSSG, S-methylglutathione, cysteinylglycine, cysteine or glutamate did not promote MgATP-dependent, uncoupler-insensitive Cd^{2+} uptake by vacuolar membrane vesicles from either strain (Table 5).

TABLE 5. Effects of different GSH-related compounds on uncoupler-insensitive ^{109}Cd uptake by vacuolar membrane vesicles purified from DTY165 or DTY167 cells. GSH, oxidized glutathione (GSSG), S-methylglutathione (GS-CH$_3$), cysteinylglycine, cysteine and glutamate were added at concentrations of 1 mM. MgATP, ^{109}CdSO$_4$ and gramicidin-D were added at concentrations of 3 mM, 80 μM and 5 μM, respectively. Values shown are means ± SE (n = 3-6).

COMPOUND	^{109}Cd UPTAKE ($nmol/mg/10$ $minutes$)			
	DTY165		DTY167	
	-MgATP	+MgATP	-MgATP	+MgATP
Cd^{2+}	5.8 ± 2.4	5.6 ± 1.5	4.3 ± 1.3	4.6 ± 2.1
Cd^{2+} + GSH	4.2 ± 1.2	37.4 ± 4.5	3.3 ± 1.1	8.3 ± 2.7
Cd^{2+} + GSSG	-	5.1 ± 3.2	-	3.8 ± 2.3
Cd^{2+} + GS-CH$_3$	-	4.5 ± 1.9	-	3.7 ± 3.1
Cd^{2+} + Cys-Gly	-	5.6 ± 3.2	-	6.9 ± 1.4
Cd^{2+} + Cys	-	7.0 ± 1.2	-	3.9 ± 1.0
Cd^{2+} + Glu	-	5.7 ± 1.1	-	5.2 ± 1.3

Purification of Transport-Active Complex

To determine the mode of action of GSH and the form in which Cd^{2+} is transported, reaction mixtures initially containing Cd^{2+} and GSH were fractionated and *YCF1*-dependent uptake was assayed.

5   Incubation of ^{109}Cd^{2+} with GSH and gel-filtration of the mixture on Sephadex G-15 yielded two major ^{109}Cd-labeled peaks: a low molecular weight peak *(LMW-Cd.GS)* and a high molecular weight peak *(HMW-Cd.GS)* (Figure 9A). When rechromatographed on Mono-Q, *LMW-Cd.GS* and *HMW-Cd.GS* eluted at 0 (Figure 9C) and 275 mM NaCl, respectively (Figure 9B). Of these two ^{109}Cd-labeled components,

10   *HMW-Cd.GS* alone, underwent *YCF1*-dependent transport. MgATP-dependent, uncoupler-insensitive *HMW-^{109}Cd.GS* uptake by DTY165 membranes increased as a single Michaelian function of concentration (K_m, 39.1 ± 14.1 μM; V_{max}, 157.2 ± 60.7 nmol/mg/10 minutes) (Figure 10A). By contrast, uptake of *LMW-^{109}Cd.GS* by DTY165 membranes was negligible at all of the concentrations examined (Figure

15   10B). Vacuolar membranes from DTY167 cells transported neither *HMW-^{109}Cd.GS* nor *LMW-^{109}Cd.GS* (Figures 10A and 10B).

Bis(glutathionato)cadmium Is the Transport-Active Complex

The transport-active complex, _HMW-Cd.GS_, was identified as

bis(glutathionato)cadmium (Cd.GS$_2$) by three criteria: (i) The average Cd:GS molar

ratio of the transported species, estimated from the ^{109}Cd:^{3}H ratios of the _HMW-Cd.GS_

peaks obtained after chromatography of reaction mixtures initially containing ^{109}Cd^{2+}

and [^{3}H]GSH on Sephadex G-15 and Mono-Q were 0.44 ± 0.09 and 0.49 ± 0.17,

respectively (Table 6). (ii) DTY165 membranes accumulated ^{109}Cd and [^{3}H]GS in a

molar ratio of 0.49 ±0.01 when incubated in media containing _HMW-^{109}Cd.[^{3}H]GS_,

MgATP and gramicidin-D (Table 6). (iii) The principal ion peak detected after

MALD-MS of _HMW-Cd.GS_ had an m/z ratio of 725.4 ± 0.7, consistent with the

molecular weight of _bis_(glutathionato)cadmium (724.6 Da, Figure 11). The transport-

inactive complex, _LMW-Cd.GS_, on the other hand, was tentatively identified as

mono(glutathionato)cadmium on the basis of its smaller apparent molecular size

(Figure 9A), failure to bind Mono-Q (Figure 9C) and Cd:GS ratio of 0.67 ± 0.04 and

0.86 ± 0.07 after chromatography on Sephadex G-15 and Mono-Q (Table 6),

respectively.

While an m/z ratio of 725 for _HMW-Cd.GS_ would be equally

compatible with the transport of Cd.GSSG, this is refuted by two findings: (i) GSSG

alone does not promote _YCF1_-dependent uptake (Table 5). (ii) The transport-active

complex is probably a mercaptide. Pretreatment of _HMW-Cd.GS_ with 2-

mercaptoethanol inhibits MgATP-dependent, uncoupler-insensitive Cd^{2+} uptake by

DTY165 membranes by more then 80% (Table 6) and _S_-methylation abolishes the

stimulatory action of GSH (Table 5).

TABLE 6. Molar Cd:GS ratios of *LMW-Cd.GS* and *HMW-Cd.GS* complexes fractionated by Sephadex G-15 and Mono-Q chromatography (Figure 9) before and after MgATP-dependent, uncoupler-insensitive uptake by vacuolar membrane vesicles purified from DTY165 and DTY167 cells. Cd:GS ratios were estimated from the ^{109}Cd:[^{3}H] radioisotope ratios of samples prepared from ^{109}CdSO$_4$ and [^{3}H]GSH. *HMW-^{109}Cd.*[^{3}H]GS was pretreated with 2-mercaptoethanol (2-ME) by heating a 1:4 mixture of *HMW-^{109}Cd.*[^{3}H]GS with 2-ME at 60°C for 10 minutes before measuring ^{109}Cd^{2+} uptake. Uptake was measured using 50 μM concentrations (as Cd) of the complexes indicated in standard uptake medium containing 5 μM gramicidin-D. Values shown are means ± SE (n = 3-6).

FRACTION	^{109}Cd UPTAKE (nmol/mg/10 min)		MOLAR RATIO Cd:GS	
	DTY165	DTY167	BEFORE UPTAKE	AFTER UPTAKE
Sephadex G-15				
HMW-Cd.GS	-	-	0.44 ± 0.09	-
LMW-Cd.GS	-	-	0.67 ± 0.04	-
Mono-Q				
HMW-Cd.GS	66.3 ± 2.7	5.6 ± 2.6	0.49 ± 0.17	0.49 ± 0.01
LMW-Cd.GS	4.4 ± 0.8	3.9 ± 1.4	0.86 ± 0.07	-
After 2-ME				
HMW-Cd.GS	11.9 ± 2.4	4.4 ± 3.0	-	-

Cd.GS$_2$ Transport is Directly Energized by MgATP

Purification of Cd.GS$_2$ enabled the energy requirements of YCF1-dependent transport to be examined directly and confirmed that more than 83% of the MgATP-dependent, uncoupler-insensitive Cd^{2+} transport measured using DTY165 membranes was mediated by YCF1. Agents that dissipate both the ΔpH and ΔΨ components of the H$^+$-electrochemical gradient established by the V-ATPase (FCCP, gramicidin-D) or directly inhibit the V-ATPase, itself (bafilomycin A$_1$), decreased MgATP-dependent Cd-GS$_2$ uptake by vacuolar membrane vesicles from DTY165 cells by 22% (Table 7). Ammonium chloride which abolishes ΔpH while leaving ΔΨ unaffected, on the other hand, inhibited uptake by only 15% (Table 7). From these results and the inability of uncouplers to markedly increase the inhibitions caused by V-ATPase inhibitors alone (Table 7), Cd.GS2 uptake by wild type membranes is

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inferred to proceed *via* a YCF1-dependent, MgATP-energized pathway that accounts for most of the transport measured and a YCF1-independent pathway, primarily driven by the H^+-gradient established by the V-ATPase, that makes a minor contribution to total uptake.

Table 7. Effects of uncouplers and V-ATPase inhibitors on uptake of *bis*(glutathionato)cadmium ($Cd.GS_2$) by vacuolar membrane vesicles purified from DTY165 and DTY167 cells. Uptake was measured in standard uptake medium containing 50 µM purified $^{109}Cd.GS_2$. Bafilomycin A_1, FCCP, gramicidin-D and NH_4Cl were added at concentrations of 0.5 µM, 5 µM, 5 µM, and 1 mM, respectively. Values outside parentheses are means ± SE (*n* = 3-6); values inside parentheses are rates of uptake expressed as percentage of control.		
	$^{109}Cd.GS_2$ UPTAKE *(nmol/mg/10 minutes)*	
ADDITION	DTY165	DTY167
Control	105.8 ± 12.4 (100)	17.3 ± 2.7 (100)
Gramicidin-D	77.8 ± 6.4 (73.5)	9.3 ± 2.0 (56.6)
FCCP	62.2 ± 11.4 (58.8)	10.2 ± 1.6 (59.0)
NH_4Cl	89.8 ± 8.2 (84.8)	10.0 ± 1.7 (57.8)
NH_4Cl+ gramicidin-D	69.8 ± 12.0 (66.0)	8.8 ± 2.2 (50.9)
Bafilomycin A_1	81.8 ± 6.0 (76.6)	12.8 ± 3.6 (74.0)
Bafilomycin A_1+ gramicidin-D	70.2 ± 12.2 (66.4)	7.2 ± 2.4 (41.6)

$Cd.GS_2$ Competes with DNP-GS for Transport

As would be predicted if $Cd.GS_2$ and the model organic GS-conjugate DNP-GS follow the same transport pathway, the K_i for inhibition of MgATP-dependent, uncoupler-insensitive Cd.GS2 uptake by DNP-GS (11.3 ± 2.1 µM; Figures 10A and 10C) coincided with the K_m for DNP-GS transport (14.1 ± 7.4 µM).

Pretreatment with Cd^{2+} or CDNB Increases *YCF1* Expression

RNase protection assays of *YCF1* expression in DTY165 cells and measurements of MgATP-dependent, uncoupler-insensitive $^{109}Cd.GS_2$ and [^3H]DNP-GS uptake by vacuolar membranes prepared from the same cells after 24 hour of growth in media containing $CdSO_4$ (200 µM) or the cytotoxic DNP-GS precursor, CDNB (150 µM), demonstrated a parallel increase in all three quantities. *YCF1-*

specific mRNA levels were increased by 1.9- and 2.5-fold by pretreatment of DTY165 cells with CdSO$_4$ and CDNB, respectively (Figure 12). The same pretreatments increased MgATP-dependent, uncoupler-insensitive [109]Cd.GS$_2$ uptake into vacuolar membrane vesicles by 1.4- and 1.7-fold and [^{3}H]DNP-GS uptake by 1.6- and 2.8-fold (Figure 12).

These investigations provide the first indication of the mechanism by which *YCF1* confers Cd^{2+} resistance in *S. cerevisiae* and its relationship to the transport of organic GS-conjugates by demonstrating that the integral membrane protein encoded by this gene specifically catalyzes the MgATP-energized uptake of *bis*(glutathionato)cadmium by vacuolar membrane vesicles.

The codependence of Cd-GS$_2$ and organic GS-conjugate transport on *YCF1* is evident at multiple levels: (i) The *ycf1*Δ mutant strain, DTY167, is hypersensitive to Cd^{2+} and CDNB in the growth medium and both hypersensitivities are alleviated by transformation with plasmid-borne *YCF1*. (ii) Vacuolar membrane vesicles purified from DTY167 cells are grossly impaired for MgATP-energized, uncoupler-insensitive organic GS-conjugate and GSH-promoted Cd^{2+} uptake. (iii) Cd.GS$_2$, and organic GS-conjugates compete for the same uptake sites on YCF1. (iv) Factors that increase *YCF1* expression elicit a parallel increase in Cd.GS$_2$ and organic GS-conjugate transport. Thus, a number of ostensibly disparate observations, the strong association between cellular GSH levels and Cd^{2+} resistance (*e.g.*, Singhal *et al.*, 1987, *FASEB J.* 1:220-223), the markedly increased sensitivity of vacuole deficient *S. cerevisiae* strains to Cd^{2+} toxicity, and the coordinate regulation of the yeast *YCF1* and *GSH1* genes, the latter of which encodes γ-glutamylcysteine synthetase (Wemmie *et al.*, 1994, *supra*; Wu *et al.*, 1994, *supra*), are now explicable in terms of a model in which YCF1 catalyzes the GSH-dependent vacuolar sequestration of Cd^{2+}.

Further, at the biochemical level, YCF1 specifically catalyzes the transport of Cd.GS$_2$ as the data provided herein establish. In addition, at the cellular level, YCF1 confers resistance to and is induced by a spectrum of xenobiotics. Expression of *YCF1* is increased by exposure of cells to glutathione-conjugable

xenobiotics and Cd^{2+}. The close resemblance of YCF1 to MRP1, the capacity of YCF1 for both organic toxin and heavy metal transport, and its discovery in one of the most tractable and thoroughly molecularly characterized eukaryotes, *S. cerevisiae*, establishes that YCF1 is useful for manipulation of the transport of organic toxins and heavy metals in plants, mammals and yeast. Thus, according to the present invention, methods for overcoming, or at least diminishing, heavy metal contamination through bioremediation using native species or genetically engineered organisms are now possible.

Cloning of plant *MRP1/YCF1* homologs

As described herein, the data presented herein establish that *YCF1* encodes a protein functionally equivalent to human MRP1. There is next described the discovery that two plant genes, *AtMRP1* and *AtMRP2*, from *Arabidopsis* encode MRP1/YCF1 homologs.

To isolate genes likely involved in glutathione *S*-conjugate transport from *Arabidopsis thaliana*, degenerate PCR primers corresponding to appropriate portions of human *MRP1* (Cole *et al.*, 1992, *supra*) and *YCF1* (Szczypka *et al.*, 1994, *supra*) were designed. Four degenerate primers were synthesized but only two of these yielded amplification products of the appropriate size that hybridized with *MRP1* and *YCF1*. The sequences of the two primers were:

5'-GARAARGTIGGIATHGTIGGIMGIACIGGIGC-3'(MRP2) (SEQ ID NO:17) and 5'-TCCATDATIGTRTTIARICKTGIGC-3'(MRP4) (SEQ ID NO:18), where I = inosine, K = T or G, M = C or A and R = A or G. MRP2 corresponds to positions 1321-1331 and 1300-1310 in *MRP1* and *YCF1*, respectively; MRP4 corresponds to positions 1486-1494 and 1466-1474. Database searches confirmed that the sequences of the peptides specified by MRP2 and MRP4 were specific to *MRP1* and *YCF1* but not any other ABC transporter in GenBank database release 90 (Altschul *et al.*, 1990, *J. Mol. Biol.*, 215: 403-410).

Degenerate PCR was performed using *Arabidopsis* genomic DNA as template. Amplification was for 45 cycles using the following thermal profile: 94°C

for 30 seconds, 50°C for 30 seconds and 72°C for 1 minute. A 0.6 kb PCR product was isolated, shown to hybridize strongly with a mixed probe encompassing the second NBF domain of *MRP1* and *YCF1*, and was cloned into pCRII vector (Invitrogen).

Sequence analysis verified that the deduced translation product of the *Arabidopsis* PCR product exhibited greatest similarity to YCF1 and MRP1 plus an unidentified 1.7 kb *Arabidopsis* EST (ATTS1246; Hofte *et al.*, 1993, *Plant J.*, 4: 1051-1061). In order to increase the likelihood of obtaining positive clones, a mixed probe consisting of the 0.6 kb PCR product and 1.6 kb EST was employed for further screens.

Eleven independent positive clones were obtained after screening approximately 3 x 10^5 plaques of a size-fractionated (3-6 kb) *Arabidopsis* cDNA library constructed in λZAPII (Kieber *et al.*, 1993, *Cell*, 72: 427-441) with the mixed probe. Restriction mapping confirmed that all 11 isolates corresponded to the same gene. The longest of these inserts, a 3.5 kb insert, designated *AtMRP2*, was subcloned and sequenced (Figure 13).

Since this isolate of *AtMRP2* was estimated to be missing approximately 1.5 kb of the 5' sequence of the ORF (assuming that the complete ORF of *AtMRP2* is similar in size to the ORFs of human *MRP1* and yeast *YCF1*), 500 bp of the most 5' sequence of *AtMRP2* was used to probe two *Arabidopsis* bacterial artificial chromosome (BAC) libraries, UCD and TAMU (Choi *et al.*, 1995, *Weeds World*, 2: 17-20) to isolate clones containing the missing sequence. This procedure yielded 8 BAC clones: U1L22, U8C12, U12A2, U23J22, U419, T9C22, T1B17 and T4K22. After digestion with *Hind*III, those fragments that hybridized with the 3.5 kb cDNA insert were introduced into pBluescript SK⁻ and sequenced. Two of these BAC clones (T1B17, T4K22) comprise a second *MPR1* plant homolog, designated *AtMRP1*, while the remainder (U1L22, U8C12, U12A2, U23J22, U419, T9C22) comprise *AtMRP2* (see below).

After establishing that an approximately 10 kb *Hind*III fragment from BAC clone U1L22 encompassed sequences identical to *AtMRP2*, a *Bgl*II restriction fragment comprising the first 3 kb of the BAC clone was used to rescreen

approximately 2×10^6 plaques from the *Arabidopsis* λZAPII cDNA library. Twenty

six independent positive clones were obtained and the one containing the longest

cDNA insert, 5.2 kb, was sequenced.

Sequence analysis demonstrated that the 5.2 kb cDNA was not identical

to *AtMRP2* but instead a very closely related gene. Designated *AtMRP1* (Figure 16),

the 5.2 kb cDNA was 84.3% and 88.2% identical to *AtMRP2* at the nucleotide and

amino acid levels, respectively. Importantly, *AtMRP1* is a full-length cDNA.

Having determined the complete sequence of the *AtMRP1* cDNA, it was

possible to identify the initiation codon of the *AtMRP2* genomic clone, design a

specific 5'-UTR primer and amplify the remaining 5' end of *AtMRP2* to generate a full-

length cDNA. Thus, full-length cDNAs encoding AtMRP2 and AtMRP1 (Figures 13

and 16, respectively) and genomic clones corresponding to *AtMRP2* and *AtMRP1* have

been generated (Figure 14 and 17, respectively). The deduced amino acid sequences of

AtMRP2 and AtMRP1 are presented in Figures 15 and 18, respectively.

Expression of *AtMRP1* in *Saccharomyces cerevisiae*

The experiments described below establish that AtMRP1 mediates the

MgATP-dependent transport of GS-conjugates. The results of similar experiments on

AtMRP2 demonstrate that this gene product has the same transport capability.

The data presented herein establishes that *YCF1* from *Saccharomyces*

cerevisiae encodes a 1,515 amino acid ATP-binding cassette (ABC) transporter protein

which localizes to the vacuolar membrane and catalyzes MgATP-dependent GS-

conjugate transport. Membrane vesicles from wild type (DTY165) cells contain two

pathways for transport of the model GS-conjugate, DNP-GS: an MgATP-dependent,

uncoupler-insensitive pathway and an electrically driven pathway. Membranes from

the mutant strains DTY167 and DTY168, harboring a deletion of the *YCF1* gene, are

by contrast more than 90% impaired in MgATP-dependent, uncoupler-insensitive

DNP-GS transport. Yeast strains lacking a functional *YCF1* gene therefore represent a

model system for probing the GS-conjugate transport function of plant *YCF1/MRP1*

homologs.

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To test the transporter capacity of *AtMRP1* (the first clone for which a full-length cDNA was obtained) for conferring GS-conjugate transport, yeast strain DTY168 (disrupted for the *YCF1* gene) was transformed with an expression vector engineered to contain the coding sequence of *AtMRP1*. After selection of the transformants, membranes were prepared and assayed for MgATP-dependent, uncoupler-insensitive DNP-GS transport as described herein. The results establish that AtMRP1 catalyzes GS-conjugate transport in a manner indistinguishable from the vacuolar GS-X pump.

Construction of the expression vector

In order to constitutively express the *AtMRP1* gene in *S. cerevisiae*, a derivative of the yeast-*E. coli* shuttle vector, pYES2 (Invitrogen), was constructed. Essentially, the 831 bp *XbaI/NotI* fragment encompassing the 3-phosphoglycerate kinase (*PGK*) promoter of plasmid pFL61 (Minet et al., 1992, Plant J. 2:417-422) was inserted between the *SpeI/NotI* restriction sites of pYES2. In so doing, the galactose-inducible yeast *GAL1* promoter of pYES2 was replaced by the constitutive yeast *PGK* promoter, *pPGK*. This plasmid, designated pYES3, is otherwise identical to pYES2. The gene to be expressed is inserted into the multiple cloning site located between the *PGK* promoter and *CYC1* termination sequences.

Preliminary experiments had established that the 5' untranslated region (UTR) of the original *AtMRP1* cDNA isolate diminished expression of the open reading frame in yeast. Thus, to maximize expression, the 127 bp 5'-UTR of *AtMRP1* was removed. For this purpose, pBluescript SK⁻-AtMRP1 was digested with *HpaI/SnaBI* to delete 3045 bp of the internal sequence. The remaining 5 kb fragment from this digest was gel-purified and self-ligated to generate truncated *AtMRP1* cDNA as a template for PCR. One hundred pmol of *AtMRP1*-Nco primer (5'-AAACCGGTGCGGCCGCCATGGGGTTTGAGCCGT-3') (SEQ ID NO:19) and 100 pmol of T3 primer (5'-AATTAACCCTCACTAAAGGG-3') (SEQ ID NO:20) were used to amplify a 2002 bp fragment of *AtMRP1* using Pfu DNA polymerase

(Stratagene). Amplification was for 30 cycles using the following thermal profile: 94°C for 15 seconds; 50°C for 15 seconds; and 72°C for 3.5 minutes.

The PCR product was gel-purified, digested with *Spe*I and cloned into the *EcoRV/Spe*I sites of pBluescript SK⁻ to generate pSK⁻ *AtMRP1*-Nco2. The 1227 bp *Sph*I/*Spe*I fragment of this construct was then exchanged with the 4363 bp *Sph*I/*Spe*I fragment of pBluescript pSK⁻ *AtMRP1* to generate pSK⁻ *AtMRP1*-Nco5. pYES-AtMRP1, lacking the 5' UTR, was constructed by digesting pSK⁻ *AtMRP1*-Nco5 with *Xho*I/*Spe*I to obtain a 5049 bp truncated *AtMRP1* gene fragment which was cloned into the *Xho*I/*Xba*I sites of pYES3. One kb of the 5' sequence of the *AtMRP1* insert of pYES3-*AtMRP1* was analyzed and was found to match exactly the sequence of the original cDNA clone.

Transformation of Yeast

S. cerevisiae strain DTY168 (MATα *his6, leu2-3, -112, ura3-52 ycf1::his*G) was transformed with pYES3-*AtMRP1* or empty vector lacking the *AtMRP1* insert (pYES3) by the LiOAc/PEG method (Giest et al., 1991, Yeast 7:253-263) and selected for uracil prototrophy by plating on AHC medium containing tryptophan (Kim et al., 1994, *supra*).

Isolation of membrane vesicles

For the preparation of membrane vesicles, 15 ml volumes of stationary phase cultures of the transformants were diluted into 1 L of fresh AHC medium and grown to an $OD_{600 \, nm}$ of about 1.2. Membrane vesicles were purified as described herein and in Kim *et al.* (1995, *supra*).

Measurement of DNP-GS uptake

DNP-GS uptake was measured as described herein in 200 μl reaction volumes containing 3 mM ATP, 3 mM MgSO₄, 5 μM gramicidin-D, 10 mM creatine phosphate, 16 units/ml creatine kinase, 50 mM KCl, 1 mg/ml BSA, 400 mM sorbitol, 25 mM Tris-Mes (pH 8.0) and the indicated concentrations of [³H]DNP-GS (17.4 mCi/mmol). Gramicidin-D (uncoupler) was included to abolish the H^+-

electrochemical potential difference that would otherwise be established by the V-ATPase in media containing MgATP.

The results of this study

Membrane vesicles purified from pYES3-*AtMRP1*-transformed DTY168 cells exhibit an approximately 4-fold increase in MgATP-dependent, uncoupler-insensitive [^{3}H]DNP-GS uptake by comparison with membrane vesicles purified from DTY168 cells transformed with empty vector (Figure 19). When measured at a DNP-GS concentration of 61.3 μM, the initial rates of uptake by membrane vesicles purified from pYES3-*AtMRP1*-transformed and pYES3-transformed cells were 0.4 nmol/mg/minute and 0.1 nmol/mg/minute, respectively (Figure 19).

The concentration dependence and vanadate inhibitility of uptake verify direct participation of AtMRP1. MgATP-dependent, uncoupler-insensitive uptake by membrane vesicles purified from the pYES3-*AtMRP1* transformants increases as a single hyperbolic function of DNP-GS concentration to yield K_m and V_{max} values of 48.7 ± 15.4 μM and 6.0 ± 1.7 nmol/mg/10 minutes, respectively (Figure 19). pYES3-AtMRP1-dependent DNP-GS uptake decreases as a single exponential function of the concentration of the phosphoryl transition state analog vanadate, to yield an I_{50} of 8.3 ± 3.3 μM (Figure 20). By contrast, the apparent K_m for DNP-GS uptake by membrane vesicles purified from pYES3-transformed DTY168 cells is in excess of 500 μM and uptake is insensitive to vanadate.

On the basis of its sequence characteristics and the results of these experiments, *AtMRP1* encodes the vacuolar GS-*X* pump. The increases in uptake following the introduction of plasmid borne *AtMRP1* into yeast (*ca.* 4 nmol/mg/20 minutes) are commensurate with the rates of MgATP-dependent, uncoupler-insensitive DNP-GS uptake measured in vacuolar membrane vesicles purified from plant sources (2.3, 3.8, 18.2, 5.8, and 2.1 nmol/mg/20 minutes for *Arabidopsis* leaf, *Arabidopsis* root, *Beta vulgaris* storage root, *Vigna radiata* hypocotyl and *Zea mays* root, respectively) (Table III in Li *et al.*, 1995, *supra*). The K_m for DNP-GS transport by heterologously

expressed AtMRP1 is similar to that reported for the endogenous GS-X pump of plant

vacuolar membranes (81.3 ± 41.8 µM, Li *et al.*, 1995, supra). The I_{50} for inhibition of

AtMRP1-dependent DNP-GS transport by vanadate coincides with the I_{50} for inhibition

of the endogenous vacuolar GS-X pump of plant cells (7.5 ± 3.9 µM, Li *et al.*, 1995,

5   *supra*).

Having confirmed that the endogenous vacuolar *GS-X* pump of *S.*

cerevisiae is lacking in the *ycf1*Δ mutant strains, DTY168 and DTY167 (Li *et al.*,

1995, *supra*), and in any case has a markedly lower K_m for DNP-GS and is 6 to 8-fold

less sensitive to vanadate than the plant cognate, these findings establish that AtMRP1

10   *per se* is responsible for the MgATP-dependent, uncoupler-insensitive transport

measured in these experiments. Given that heterologous expression of *AtMRP1* alone

is sufficient for DNP-GS transport in DTY168 cells, it is concluded that one of the GS-

X pumps of *Arabidopsis* has been cloned in its entirety.

Sequence comparisons of MRP1, cMOAT, YCF1, AtMRP1 and

15   AtMRP2 with other members of the ABC transporter superfamily reveal two major

subgroups. One group contains MRP1, cMOAT, YCF1, AtMRP1, AtMRP2 and the

Leishmania P-glycoprotein-related molecule (Lei/PgpA). The other group contains the

MDRs, the major histocompatibility complex transporters and STE6. However, of all

the ABC transporters defined to date, cMOAT, YCF1, AtMRP1 and AtMRP2 exhibit

20   the closest resemblance to each other. Unlike the similarities between the GS-X pump

subgroup, Lei/PgpA and CFTR, which center on the nucleotide binding folds (NBFs),

the similarities between the GS-X pump members cMOAT, YCF1, AtMRP1 and

AtMRP2 are found throughout the sequence. GS-X family members are 40-45%

identical (60-65% similar) at the amino acid level, possess NBFs with an equivalent

25   spacing of conserved residues and are colinear with respect to the location, extent and

alteration of putative transmembrane spans and extramembrane domains. Two features

of members of the GS-X pump family that distinguish them from other ABC

transporters are their possession of a central truncated CFTR-like regulatory domain

rich in charged amino acid residues and an approximately 200 amino acid residue N-terminal extension.

A hydropathy aligment of AtMRP1, AtMRP2, YCF1, HmMRP1, and RtCMOAT is shown in Figure 21. Note the following: (i) The almost exact equivalence of AtMRP1 and AtMRP2 with respect to the alternation of hydrophobic and hydrophilic stretches. (ii) The close correspondence of AtMRP1 and AtMRP2 with all of the other members of the MRP1/YCF1/cMOAT subclass of ABC transporters in terms of the overall hydropathy profiles. (iii) The "signature" profile for the N-terminal 200 amino acid residues of all of the sequences shown, which is unique to the MRP1/YCF1/cMOAT subclass. Hydropathy was computed according to Kyte and Doolittle (1982, *J. Mol. Biol.* 46:105-132) over a running window of 15 amino acid residues. Hydrophobic stretches of sequence fall below the line and hydrophilic stretches fall above the line.

In Figure 22 there is depicted domain comparisons between AtMRP1, ScYCF1, HmMRP1, RtCMOAT, RbEBCR and HmCFTR. The domains indicated are the N-terminal extension (NH_2), first and second transmembrane spans (TM1 and TM2, respectively), first and second nucleotide binding folds (NBF1 and NBF2, respectively), putative CFTR-like regulatory domain (R), and the C-terminus (COOH). This comparison is also tabulated in Tables 8 and 9.

TABLE 8: Identity and similarity analysis of putative domains of AtMRP1 against AtMRP2 ScYCF1, HmMRP1, RtCMOAT, HmCFTR and RbEBCR. ScYCF1, *Saccharomyces cerevisiae* YCF1; HmMRP1, human MRP1; RtCMOAT, rat cMOAT; HmCFTR, human CFTR; RbEBCR, rabbit EBCR. The domains identified are N-terminal extension (NH_2), transmembrane segments 1 and 2 (TM1 and TM2, respectively), CFTR-like regulatory domain (R), nucleotide binding folds 1 and 2 (NBF1 and NBF2, respectively) and C-terminus (COOH). Similarity was calculated as described herein over the sequence segments indicated in Table 9.					
SEQUENCE	DOMAIN	OVERALL	NH_2	TM1	NBFI
AtMRP2	*Identity*	87.0	74.4	90.4	92.1
	Similarity	93.7	85.2	96.1	96.7
ScYCF1	*Identity*	36.1	13.3	32.2	50.0

SEQUENCE	DOMAIN	OVERALL	NH₂	TM1	NBFI
	Similarity	55.4	32.9	52.6	75.0
HmMRP1	*Identity*	41.5	16.2	37.4	58.0
	Similarity	63.3	34.8	57.5	78.7
RtCMOAT	*Identity*	38.6	19.6	33.8	58.7
	Similarity	60.2	36.7	61.0	80.0
HmCFTR	*Identity*	29.2	0	22.8	40.7
	Similarity	55.1	0	47.8	62.0
RbEBCR	*Identity*	38.9	17.2	34.5	62.4
	Similarity	60.4	34.9	61.6	82.6
SEQUENCE	DOMAIN	R	TM2	NBF2	COOH
AtMRP2	*Identity*	80.5	86.9	91.3	89.4
	Similarity	89.8	94.2	96.5	94.4
ScYCF1	*Identity*	33.9	34.7	34.7	58.1
	Similarity	59.5	57.9	57.9	71.8
HmMRP1	*Identity*	31.6	31.9	61.9	48.3
	Similarity	50.4	56.3	72.8	69.0
RtCMOAT	*Identity*	33.9	34.4	60.7	50.0
	Similarity	50.0	58.8	75.7	67.2
HmCFTR	*Identity*	45.7	22.3	39.5	28.1
	Similarity	75.0	51.3	61.6	58.4
RbEBCR	*Identity*	35.6	34.1	61.9	43.0
	Similarity	51.7	59.1	74.0	62.7

TABLE 9.	Positions and sizes of segments of sequence analyzed in Table 8.				
SEQUENCE	DOMAIN	OVERALL	NH₂	TM1	NBF1
AtMRP1	*Position*		1-223	224-631	634-782
	Size	1622	223	407	148
AtMRP2	*Position*		1-223	224-631	634-782

SEQUENCE	DOMAIN	OVERALL	NH₂	TM1	NBF1
	Size	1622	223	407	148
ScYCF1	Position		1-210	211-645	646-787
	Size	1515	210	435	142
HmMRP1	Position		1-240	241-660	661-810
	Size	1531	240	420	150
RtCMOAT	Position		1-192	193-648	649-799
	Size	1540	192	456	151
HmCFTR	Position		0	1-440	441-590
	Size	1481	0	440	150
RbEBCR	Position		1-193	194-651	652-800
	Size	1562	193	458	149

SEQUENCE	DOMAIN	R	TM2	NBF2	COOH
AtMRP1	Position	783-900	901-1244	1245-1417	1418-1622
	Size	117	343	172	205
AtMRP2	Position	783-905	906-1249	1250-1422	1423-1622
	Size	122	343	172	200
ScYCF1	Position	788-936	937-1279	1280-1453	1454-1515
	Size	149	343	174	163
HmMRP1	Position	811-960	961-1300	1301-1473	1474-1531
	Size	150	340	173	59
RtcMOAT	Position	800-960	961-1302	1303-1476	1477-1541
	Size	161	342	174	65
HmCFTR	Position	591-847	848-1217	1218-1389	1390-1481
	Size	256	371	172	92
RbEBCR	Position	801-961	962-1304	1305-1477	1478-1562
	Size	161	343	173	86

As is apparent from the data presented above, there is significant homology between similar domains among AtMRP-related proteins. In particular, the N-terminal and R domains share significant homology among the AtMRP-related proteins tested. These data establish that in addition to primary sequence, the secondary structure of the molecule plays a significant role in GS-X pump function.

It should be appreciated that *AtMRP1* and *AtMRP2* constitute a family of genes in *Arabidopsis*, wherein various members of the family have different substrate specificities as demonstrated by the next set of experiments.

<u>Substrate Preferences of AtMRP1 and AtMRP2</u>

To examine the substrate preferences of AtMRP1 and AtMRP2, the following experiments were performed.

<u>Isolation of *Bn*-NCC-1</u>

[^{14}C]*Bn*-NCC-1 (33.3 mCi/mmol) was extracted from senescent cotyledons of rape (*Brassica napus*) and was purified by preparative HPLC (Krautler *et al.*, 1992, *Plant Physiol. Biochem.* 30:333-346). Determination of the purity of the final preparation by analytical HPLC and enumeration of concentration and specific radioactivity (33.3 mCi/mmol) were performed according to Hinder *et al.* (1966, *J. Biol. Chem.* 271:27233-27236). Unlabeled *Bn*-NCC-1 was isolated from fully senescent cotyledons of excised shoots that had been maintained in complete darkness for 1 week.

<u>Measurement of transport</u>

Cells were grown and vacuolar membrane-enriched vesicles were prepared as described (Kim *et al.*, 1995, *J. Biol. Chem.* 270:2630-2635-). Uptake of [^{14}C]*Bn*-NCC-1, [^{3}H]C3G-GS, [^{3}H]DNP-GS, [^{3}H]GSSG, [^{14}C]metolachlor or [^{3}H]taurocholate was measured routinely in 200 μl reaction volumes containing membrane vesicles (10-20 μg protein), 3 mM ATP, 3 mM MgSO$_4$, 5 μM gramicidin-D, 10 mM creatine phosphate, 16 units/ml creatine phosphate kinase, 50 mM KCl, 1mg/ml BSA, 400 mM sorbitol, 25 mM Tris-Mes (pH 8.0) and the indicated

concentrations of transport substrate. Uptake was terminated by the addition of 1 ml
ice-cold wash medium (400 mM sorbitol/3 mM Tris-Mes, pH 8.0) and vacuum
filtration of the suspension through prewetted Millipore HA cellulose nitrate filters
(pore size 0.45 μm). The filters were rinsed twice with wash medium, and the retained

5   radioactivity was determined by liquid scintillation counting. Nonenergized uptake
was estimated by the same procedure except that ATP was omitted from the uptake
medium.

The effect of taurocholate on the release of [³H]DNP-GS from
membrane vesicles that had been allowed to mediate AtMRP2-dependent accumulation

10   of this compound during a preceding uptake period was determined. This was
accomplished by rapid depletion of ATP from the uptake medium using a hexokinase
trap (glucose + ATP -> glucose-6-phosphate + ADP) and measurements of the decrease
in vesicular radiolabel in the presence or absence of taurocholate. Membranes from
DTY168/pYES3-AtMRP2 cells were incubated for 10 minutes in standard uptake

15   medium containing 61.3 μM [³H]DNP-GS after which time 200 mM glucose and 50
units/ml hexokinase (Type F-300 from baker's yeast) were added. After incubation for
a further 2 minutes, taurocholate (50 μM) or Triton X-100 (9.01% v/v) was added and
release of vesicular [³H]DNP-GS was measured as described. Control samples were
treated identically except that no additions were made after the initial 10 minute

20   incubation period.

Substrate Preferences

The absence of AtMRP2-dependent transport from DTY168 and
DTY168/pYES3 membranes and the selective inhibition of this system by micromolar
concentrations of vanadate, established that AtMRP2-dependent transport may be

25   measured in two ways. This may be accomplished by assessing the difference between
the rates of MgATP-dependent, uncoupler-insenstitive uptake by DTY168/pYES3-
AtMRP2 membranes by comparison with DTY168 or DTY168/pYES3 membranes, or
by assessing the vanadate-sensitive component of MgATP-dependent, uncoupler-
insensitive uptake by DTY168/pYES3-AtMRP2 membranes. Because the results were

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qualitatively and quantitatively similar whichever method was used, "AtMRP2-dependent" transport as used in this section refers to uptake which is measured as the increment consequent on transformation of DTY168 cells with pYES3-AtMRP2 *versus* pYES3.

5   Application of this methodology to vacuolar membrane-enriched vesicles purified from pYES3-AtMRP2- *versus* pYES3-transformed DTY168 cells and expansion of the transport assays to measurements of the concentration dependence of [^{3}H]DNP-GS, [^{3}H]GSSG, [^{14}C]metolachlor-GS, [^{14}C]Bn-NCC-1 and [^{3}H]taurocholate uptake, demonstrated that the substrate preferences and maximal transport capacities of
10   AtMRP2 and AtMRP1 differed markedly. While uptake of all of the GSH derivatives examined conformed to Michaelis-Menten kinetics, the V_{max} values for AtMRP2-dependent uptake were consistently serveralfold greater than those for AtMRP1-dependent uptake. The V_{max} values for AtMRP2-dependent uptake of [^{3}H]DNP-GS, [^{3}H]GSSG and [^{14}C]metolachlor-GS were 16.3 ± 3.1, 38.1 ± 3.2 and 136.0 ± 28.1
15   nmol/mg/10 min, respectively; the corresponding values for AtMRP1 were 8.2 ± 1.6, 6.8 ± 1.1 and 17.5±5.2 nmol/mg/10 min. With the exception of [^{3}H]GSSG whose K_m for AtMRP1-dependent uptake (21.9.2 ± 58.3 μM) was three times greater than that for AtMRP2-dependent uptake (73.0 ± 15.1μM), the K_m values estimated for AtMRP2 and AtMRP1 were very similar (65.7±29.8 *versus* 63.6 ± 36.5 μM for metolachlor-GS).

20   Single concentration (50 μM) measurements of uptake of the glutathionated anthocyanin, cyanin-3-glucoside-GS (C3G-GS), demonstrated an approximately 6-fold greater capacity of AtMRP2 for transport of this compound (rate = 48.4 ± 2.2 nmol/mg/10 min) by comparison with AtMRP1 (rate = 7.9 ± 0.7 nmol/mg/10min).

25   In no case was MgATP-dependent, uncoupler-insensitive uptake of the unconjugated precursors of the GS-compounds, DNP, GSH, metolachlor and C3G detectable.

Neither AtMRP2 nor AtMRP1 catalyzed the uptake of [^{3}H]taurocholate. Transformation of DTY168 cells with either pYES3-AtMRP2 or pYES3-AtMRP1

conferred little or no increase in the capacity of vacuolar membrane-enriched vesicles for [^{3}H]taurocholate uptake over that measured with vesicles prepared from pYES3-transformed cells. The results of these experiments are presented in Table 10.

Table 10. Kinetic parameters for uncoupler-insensitive AtMRP1- and AtMRP2-dependent transport of GS-derivatives, Bn-NCC-1 and taurocholate.

Compound	AtMRP1		AtMRP2	
	K_m	V_{max}	K_m	V_{max}
DNP-GS	73.8 ± 18.8	8.2 ± 1.6	65.7 ± 29.8	16.3 ± 3.1
GSSG	219.2 ± 58.3	6.8 ± 1.1	73.0 ± 15.1	38.1 ± 3.2
Metolachlor-GS	63.6 ± 36.5	17.5 ± 5.2	75.1 ± 31.6	136.0 ± 28.1
Bn-NCC-1	------Linear------		15.2 ± 2.3	63.1 ± 2.5
Taurocholate	------Linear------		-----Linear------	

MgATP-dependent, uncoupler-insensitive uptake by DTY168/pYES3-AtMRP1, DTY168/pYES3-AtMRP2 and DTY168/pYES3 membranes was measured as described herein. The K_m and V_{max} values were estimated by fitting the data to a single Michaelis-Menten function by nonlinear least squares analysis. Values shown are means ± SE.

The 2- to 8-fold greater capacity of AtMRP2 versus AtMRP1 for transport of the compounds examined was not attributable to differences in the levels of expression of their cDNAs from the PGK gene promoter of pYES3. Quantitative RT-PCR of equivalent amounts of total RNA extracted from DTY168/pYES3-AtMRP2 and DTY168/pYES3-AtMRP1 cells yielded similar levels of the 800 bp PCR

amplification product predicted from the sequences of the oligonucleotide primers used. Since neither amplification product was generated when PCR was performed without reverse transcription or when total RNA from DTY168/pYES3 cells was employed as template, contamination by genomic DNA and/or RT-PCR of transcripts other than those from *AtMRP2* or *AtMRP1*, respectively, was not responsible for the observed results.

Anomalous interactions between candidate transport substrates

Two critical properties of AtMRP2 were its capacity for the simultaneous transport of GS-conjugates and *Bn*-NCC-1 and its pronounced sensitivity to inhibition by taurocholate. Simultaneous measurements of [^{14}C]*Bn*-NCC-1 and [^{3}H]DNP-GS uptake by membrane vesicles purified from DTY168/pYES3-AtMRP2 cells revealed parallel accumulation of both compounds with little or no interference of the transport of one by the other. AtMRP2-dependent uptake of [^{14}C]*Bn*-NCC-1 at an extravesicular concentration equivalent to its K_m value (15μM,) was nearly three times less sensitive to DNP-GS than would be predicted if this GS-conjugate were a competitior. If DNP-GS were a simple competitive inhibitor such that its K_m value (66μM) approximated its K_i value for the inhibition of *BN*-NCC-1 uptake, 120 μM DNP-GS would be expected to inhibit [^{14}C]*Bn*-NCC-1 uptake by 48% but this was not observed. DNP-GS concentrations in excess of 120 μM decreased [^{14}C]*Bn*-NCC-1 uptake by less than 18%. Reciprocally, the concentration-dependence of AtMRP2-mediated [^{3}H]DNP-GS uptake was not affected appreciably by *Bn*-NCC-1. The K_m and V_{max} values for AtMRP2-dependent [^{3}H]DNP-GS uptake in the presence of 15 μM *Bn*-NCC-1 (80.5 ± 28.6 μM and 18.3 ± 1.6 nmol/mg/10/min) were similar to those measured in its absence.

Although neither AtMRP2 nor AtMRP1 transported taurocholate, AtMRP2-mediated transport was selectively inhibited by this compound. AtMRP1-dependent [^{3}H]DNP-GS uptake was relatively insensitive to taurocholate (I_{50} > 250 μM) but the uptake of both [^{3}H]DNP-GS and [^{14}C]*Bn*-NCC-1 by AtMRP2 was strongly inhibited ($I_{50(DNP-GS\ uptake)}$ = 27 ± 1.3 μM; $I_{50(Bn-NCC-1\ uptake)}$ = 49.5 ± 0.3 μM).

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Taurocholate at the concentrations employed appeared to exert is effect on AtMRP2-mediated transport by inhibiting pump activity directly rather than by increasing background membrane permeability and decreasing net influx by increasing passive DNP-GS efflux. Addition of taurocholate at a concentration (50 μM) sufficient to inhibit AtMRP2-dependent [^{3}H]DNP-GS uptake by 70% to DTY168/pYES3-AtMRP2 vesicles that had accumulated [^{3}H]DNP-GS for 10 minutes before arresting pump action by ATP depletion using a hexokinase trap, did not accelerate the efflux of intravesicular ^{3}H-label over that measured on vesicles subject to a hexokinase trap in the absence of taurocholate. Imposition of a hexokinase trap and addition of a concentration of detergent (Triton X-100; 0.01% v/v) known to permeate these membranes (Zhen et al., 1997, J. Biol. Chem. 272:22340-22348), on the other hand, increased the rate and extent of release of the [^{3}H]DNP-GS accumulated during the preceding 10 minute uptake period by more than 3-fold versus DTY168/pYES3-AtMRP2 vesicles treated with hexokinase alone or hexokinase plus taurocholate.

The high capacity of AtMRP2 for the transport of large amphipathic anions other than GS-conjugates (i.e., Bn-NCC demonstrates that one pump can assume more than one of the several ABC transporter-like functions identified in plants to date. In the case of AtMRP2, this includes transport activity directed to a broad-range GS-conjugate pump and a chlorophyll metabolite pump. Thus, one the one hand, the high capacity of heterologously expressed AtMRP2, and to a lesser extent AtMRP1, for the transport of metolachlor-GS, and by extension GS-conjugates of other herbicides to glutathionation, is consistent with the molecular identification of transporters capable of removing these and related compounds from the cytosol. On the other hand, the high capacity of AtMRP2 for the transport of Bn-NCC is consistent with the identification of an element capable of contributing to the further metabolism and eventual removal of tetrapyrrole derivatives generated during leaf senescence from the cytosol.

Vacuolar uptake of glutathionated medicarpin by the glutathione conjugate pump

A key event in the disease resistance response of legumes is the rapid and localized accumulation of isoflavonoid phytoalexins. Accordingly, most studies of plant-pathogen interactions in the Leguminosae have centered on the enzymology and molecular biology of the isoflavonoid biosynthetic pathway (Dixon *et al.*, 1995,

5   *Physiol. Plant* 93:385). However, the mechanism and sites of intracellular accumulation of these compounds is not understood. Since many isoflavonoid phytoalexins are as toxic to the host plant as they are to its pathogens, it is essential that they are accumulated in the plant in a site which is sequestered (*i.e.*, isolated) from the cytoplasm.

10   The following experiments describe uptake of free [³H]medicarpin by vacuolar membrane vesicles purified from etiolated hypocotyls of mung bean (*Vigna radiata*). This uptake is slow and relatively insensitive to MgATP. However, after incubation with glutathione and a total glutathione-*S*-transferase preparation from maize (*Zea mays*), [³H]medicarpin uptake occurs at a rate which is 8-fold faster in the

15   presence, as opposed to the absence of MgATP. MgATP-dependent uptake of glutathione/glutathione-*S*-transferase pretreated [³H]medicarpin is only slightly inhibited by uncoupler (gramicidin D), but is strongly inhibited by vanadate and the model glutathione-*S*-conjugate, *S*-(2,4-dinitrophenyl)glutathione. These results demonstrate that the MgATP-energized glutathione-conjugate pump identified herein

20   in the membrane preparation is capable of high affinity, high capacity transport of glutathionated isoflavonoid phytoalexins. The experimental procedures and results of these experiments are now described.

Preparation of [³H]medicarpin

[³H]medicarpin was produced by base-catalyzed tritium exchange from

25   ³H₂O using unlabeled medicarpin isolated from fenugreek (*Trigonella foenumgraecum*) seedlings exposed to 3 mM $CuCl_2$.

GST purification and conjugation of medicarpin

Two-week old maize (*Zea mays*) B73N seedlings were grown under
continuous light at 21 °C. Twenty four hours prior to harvesting, the seedlings were
exposed to a mild treatment with 2,4-dichlorophenoxyacetic acid and atrazine to
5   stimulate GST expression (Timmerman, 1989, *Physiol. Plant* 77:465). Two-gram
samples of root and shoot tissue were ground to homogeneity in 50 ml of 500 mM
sodium phosphate buffer, pH 7.8 (Buffer A). The extract was centrifuged at 7,000 x g
for 10 minutes at 4°C and the resulting supernatant was filtered through Miracloth and
mixed with 2 ml of *S*-hexylglutathione-conjugated agarose beads (Sigma). After
10 incubation for 5 minutes at 21 °C, the beads were sedimented by centrifugation at 500 x
g at 4°C. The supernatant was discarded and the beads were resuspended in 2.5 ml of
prechilled Buffer A and centrifuged again. Bound GST was eluted by resuspension of
the beads in 2 ml Buffer B (20 mM GSH, 500 mM sodium phosphate, pH 7.8) and
incubation for 5 minutes at 21 °C. The beads were sedimented by centrifugation at 500
15 x g and the supernatant was assayed for GST activity (Mannervick *et al.*, 1981,
Methods Enzymol., 77:231).

[^{3}H]medicarpin (0.5 µCi, 4.5 Ci/mol) was conjugated with GSH by
incubation with 25 µl of total purified maize GSTs for 3 hours at 21 °C in the dark.
Control, unconjugated samples were prepared by mixing [^{3}H]medicarpin (0.5 µCi)
20 with cold Buffer B and immediately freezing the mixture in liquid nitrogen.

Synthesis of S-(2,4-dinitrophenyl)glutathione (DNP-GS)

DNP-GS was synthesized from 1-chloro-2,4-dinitrobenzene and GSH
by a modification of the enzymatic procedure of Kunst *et al.*, (1989, *Biochim. Biophys.
Acta* 983:123; Li *et al.*, 1995, *supra*).

25 Preparation of vacuolar membrane vesicles

Vacuolar membrane vesicles were purified from etiolated hypocotyls of
V. radiata cv. Berken as described (Li *et al.*, *Plant Physiol.* 109: 1257, Li *et al.*, 1995,
supra).

Measurement of uptake

Unless otherwise indicated, [^{3}H]medicarpin or [^{3}H]medicarpin-GS
uptake was measured at 25°C in 200 μl reaction volumes containing 3 mM ATP, 3
mM MgSO$_4$, 10 mM creatine phosphate, 16 U/ml creatine kinase, 50 mM KCl, 0.1%
(w/v) BSA, 400 mM sorbitol and 25 mM Tris-Mes buffer, pH 8.0. Uptake was

5   initiated by the addition of 12 μl membrane vesicles (30-40 μg protein) and brief
mixing of the samples on a vortex mixer. Uptake was terminated by the addition of 1
ml ice-cold wash medium (400 mM sorbitol, 3 mM Tris-Mes, pH 8.0) and vacuum
filtration of the suspension through prewetted HA cellulose nitrate filters (pore
diameter 0.45 μm). The filters were rinsed twice with a 1 ml ice-cold wash medium,

10   air-dried and radioactivity was determined by liquid scintillation counting.

Protein estimations and source of commercial chemicals was as
described herein.

Results

Appreciable MgATP-dependent uptake of [^{3}H]medicarpin by vacuolar

15   membrane vesicles purified from etiolated hypocotyls of mung was dependent on
preincubation of this compound with GSH and GSTs. Free [^{3}H]medicarpin incubated
in the presence of GSH in the absence of GSTs was taken up at 16.7 ± 3.6 and 7.4 ± 1.3
nmol/mg/20 minutes in the presence and absence of MgATP, respectively (Figure 25).
In contrast, [^{3}H]medicarpin-GS synthesized by incubating [^{3}H]medicarpin with GSH in

20   the presence of affinity-purified maize GSTs, was taken up at 81.0 ± 13.3 and 11.3 ±0.4
nmol/mg/20 minutes in the presence and absence of MgATP, respectively (Figure 25).

MgATP-dependent [^{3}H]medicarpin-GS uptake was strongly inhibited
by vanadate and DNP-GS but was relatively insensitive to uncouplers. Whereas
inclusion of vanadate (10 μM) or DNP-GS (100 μM) in the assay medium inhibited

25   [^{3}H]medicarpin uptake by more than 85%, addition of the ionophore, gramicidin D,
diminished uptake by only 17% (Table 11).

Table 11. Effects of different inhibitors on [^{3}H]medicarpin-GS uptake by vacuolar membrane vesicles. [^{3}H]medicarpin-GS was added at a concentration of 65 μM. MgATP was either omitted (-MgATP) or added at a concentration of 3 mM (+MgATP). Gramicidin-D, vanadate and DNP-GS were added at concentrations of 5 μM, 10 μM and 100 μM, respectively. Values outside parentheses are means ± SE (n = 3); values inside parentheses are rates of uptake expressed as percentage of control.

TREATMENT	[^{3}H]Medicarpin-GS Uptake (*nmol/mg/10 minutes*)	
	+MgATP	-MgATP
Control	85.6 ± 13.3 (100)	16.7 ± 6.0 (100)
+ Gramicidin-D	71.2 ± 3.0 (83.2)	13.2 ± 2.1 (79.0)
+ Gramicidin-D + vanadate	12.9 ± 0.9 (15.1)	17.4 ± 0.5 (104.2)
+ Gramicidin-D + DNP-GS	11.7 ± 2.8 (13.7)	5.6 ± 3.1 (33.5)

MgATP-dependent, uncoupler-insensitive uptake increases as a single Michaelian function of [^{3}H]medicarpin-GS concentration to yield K_m and V_{max} values of 21.5 ± 15.5 μM and 77.8 ±23.3 nmol/mg/20 minutes, respectively (Figure 26).

Direct involvement of the GS-X pump in the accumulation of [^{3}H]medicarpin-GS by vacuolar membrane vesicles is therefore evident at three levels: (i) Glutathionation of medicarpin selectively increases MgATP-dependent uptake. MgATP-independent uptake is marginally affected by glutathionation but MgATP-dependent uptake is stimulated by approximately six-fold confirming that medicarpin-GS is the transported species and MgATP is the energy source. (ii) Uptake is directly energized by MgATP. The inability of uncoupler to markedly inhibit [^{3}H]medicarpin-GS uptake implies that the H$^+$-electrochemical gradient that would otherwise be established by the vacuolar H$^+$-ATPase in the presence of MgATP does not drive uptake. Rather, the pronounced inhibition of MgATP-dependent uptake exerted by vanadate agrees with the notion that GS-X-mediated uptake is strictly dependent on ATP hydrolysis and formation of a phosphoenzyme intermediate (Martinoia *et al.* 1993, *supra*; Li *et al.*, 1995, *supra*), (iii) [^{3}H]medicarpin-GS and the model GS-X pump substrate DNP-GS, whose transport has been exhaustively analyzed in this system as

described herein, compete for uptake indicating that both are transported by the same moiety.

The efficacy of medicarpin-GS as a substrate for the vacuolar GS-X pump is striking. Even though the K_m for medicarpin-GS uptake is undoubtedly an overestimate, since the yield from the conjugation reaction was not enumerated, it is nevertheless 2 to 25-fold lower than those estimated previously for DNP-GS, C3G-GS (80 and 46 µM in this system), glutathione-S-N-ethylmaleimide (500 µM) and metolachlor-GS (40-60 µM, barley vacuoles; Martinoia *et al.*, 1993, *supra*). Moreover, the capacity of the GS-X pump for medicarpin-GS uptake is high (V_{max} = 78 nmol/mg/20 minutes) versus DNP-GS (V_{max} = 12 nmol/mg/20 minutes) and comparable to that estimated for C3G-GS (V_{max} = 45 nmol/mg/minute). Thus, while maize anthocyanin was the first natural substrate shown to be vacuolarly sequestered through the concerted actions of cystolic GSTs and the vacuolar GS-X pump in plants (Marrs *et al.*, 1995, *Nature*, 375:397 and data contained herein), medicarpin, and presumably other isoflavonoid phytoalexins, is equally strong a candidate.

These data suggest that the GSTs which are induced following the hypersensitive response to avirulent fungal pathogens likely serve to facilitate the vacuolar storage of antimicrobial compounds in the healthy cells surrounding the lesion.

Transport of glutathionated anthocyanins and auxins by the vacuolar GS-X pump of plant cells

The data which are now described demonstrate that the vacuolar GS-X pumps of corn (*Zea mays*) roots and etiolated hypocotyls of mung bean (*Vigna radiata*) transport the anthocyanin cyanidin-3-glucoside (C3G), and the phytohormone, indole-3-acetic acid (IAA), after conjugation with glutathione. Whereas the unconjugated forms of these compounds undergo negligible uptake into vacuolar membrane vesicles, both C3G-GS and IAA-GS are subject to high rates of MgATP-dependent, uncoupler-insensitive uptake (Figure 27 and Table 12). IAA-GS and C3G-GS uptake approximates Michaelis-Menten kinetics to yield K_m values in the micromolar range

and V_{max} values 7- to 40-fold greater than those measured with the artificial transport

substrate, DNP-GS (Table 12 and Li *et al.*, 1995, *supra*). Uptake of both conjugates is

inhibited by DNP-GS and vanadate in a manner consistent with mediation by the GS-X

pump (Figure 27 and Table 13). In contrast, glutathionated abscissic acid (ABA-GS) is

5   a poor substrate for the GS-X pump: uptake is relatively slow and only saturates at high

substrate concentrations (Figure 27 and Table 13).

Table 12. Summary of kinetic parameters for MgATP-dependent, uncoupler-insensitive uptake of C3G-GS, IAA-GS and ABA-GS by vacuolar membrane vesicles purified from etiolated hypocotyls of *V. radiata* and roots of *Z. mays*. Kinetic parameters (K_m, μM; V_{max}, *nmol/mg/10 min*) were computed from the data shown in Figure 27 by nonlinear least squares analysis. Values shown are means ± SE.			
	C3G-GS		IAA-GS
PARAMETER	*V. radiata*	*Z. mays*	*V. radiata*
K_m	45.7 ± 14.0	39.5 ± 16.6	36.0 ± 16.7
V_{max}	45.3 ± 6.5	79.1 ± 14.7	17.7 ± 5.8
	IAA-GS	ABA-GS	
PARAMETER	*Z. mays*	*V. Radiata*	*Z. mays*
K_m	47.7 ± 19.6	>1000	128.8 ± 79.1
V_{max}	30.0 ± 4.9	22.9 ± 9.2	4.0 ± 1.4

It has been known for some time that the characteristic bronze

coloration of *Bronze-2* (*bz2*) mutants is a consequence of the accumulation of cyanidin-

3-glucoside in the cytosol. In wild type (*Bz2*) plants, anthocyanins are transported into

the vacuole and become purple or red whereas in *bz2* plants, anthocyanin is restricted

5   to the cytoplasm where it is oxidized to a brown ("bronze") pigment. However, until

the present invention, the exact molecular basis of this lesion was unknown. Since *Bz2*

encodes a GST responsible for conjugating anthocyanin with GSH (Marrs *et al.*, 1995)

and glutathionated anthocyanins are transported by the vacuolar GS-X pump, the

experiments described herein explain the bronze phenotype. Being defective in the glutathionation of anthocyanins, *bz2* mutants are unable to pump these pigments from the cytosol into the vacuole lumen; a conclusion borne out by the ability of the GS-*X* pump inhibitor, vanadate, to phenocopy the *bz2* lesion in wild type protoplasts and the efficacy of cyanidin-3-glucoside-GS as a substrate for the plant vacuolar GS-*X* pump *in vitro*, as the data presented herein establish.

The concept underlying the above-described experiments on phytohormones is that they may be subject to metabolic interconversions and compartmentation analogous to those deduced for anthocyanins. On the one hand, it is now established that C3G must be glutathionated before it can be transported into the vacuole. On the other hand, it is evident that most of the vacuolar anthocyanins of intact plants are not stored in this form. Instead, they are subject to long term storage as their malonyl derivatives. It is therefore apparent that while C3G-GS is a short-lived but necessary intermediate for vacuolar anthocyanin compartmentation, it is not the terminal product of this process. The experiments with auxins further illustrate this principle by demonstrating that IAA is susceptible to glutathionation and that the resultant IAA-GS conjugate is transported by the vacuolar GS-*X* pump in a MgATP-dependent, uncoupler-insensitive, vanadate-inhibitible manner. Thus, even though IAA-GS derivatives have not been detected *in planta,* this does not exclude the possibility that they are short-lived transport intermediates necessary for subsequent vacuolar processing of this class of compounds.

Table 13. Concentrations of DNP-GS and vanadate required to inhibit MgATP-dependent, uncoupler-insensitive uptake of C3G-GS, IAA-GS and ABA-GS by 50% (I_{50} values) by vacuolar membrane vesicles purified from etiolated hypocotyls of _V. radiata_ and roots of _Z. mays_. I_{50} values (μM) were estimated by nonlinear least squares analysis after fitting the data to a single negative exponential. ND, not determined.			
	C3G-GS	IAA-GS	ABA-GS
COMPOUND	_V. radiata_	_Z. mays_	_V. radiata_
Vanadate	7.9	8.2	6.5
DNP-GS	103.5	112.4	124.2
	C3G-GS	IAA-GS	ABA-GS
COMPOUND	_Z. mays_	_V. radiata_	_Z. mays_
Vanadate	5.5	ND	>150
DNP-GS	109.8	ND	231.5

Generation of a Transgenic Plant Comprising a Transgene Encoding a GS-X Pump

        To generate a transgenic plant comprising a gene encoding YCF1, the following experiments were performed. The binary vector pROK-_YCF1_, encoding

5   wild type YCF1 was constructed. The sense orientation of the inserts with respect to the CaMV 35S promoter of pROK (Baulscombe _et al._, 1986, _Nature_ 321:446-449) was confirmed and these constructs, as well as empty vector (pROK) controls, were transformed into _Agrobacterium_ strain C58 by electroporation (Ausubel _et al._, 1992, _Current Protocols in Molecular Biology_, pp 27-28).

10   Kanamycin-resistant _Agrobacterium_ transformants were isolated, the integrity of the constructs in the bacterial recipient was established by PCR and _Arabidopsis_ roots were inoculated with the transformants (Huang _et al._, 1992, _Plant Mol. Biol._ 10:372-384). The resulting rosette shoots generated on selective medium

were transferred to root-inducing medium for regeneration. Stable insertion of the sense strands and constitutive expression of *YCF1* and *YCF1::HA* was demonstrated in the kanamycin-resistant *Arabidopsis* transformants, by probing Southern blots with *YCF1* and pROK sequences and by Northern analyses, respectively.

An association between *YCF1* expression and altered xenobiotic resistance was tested by screening multiple T2 generation pROK-*YCF1*, pROK-*YCF1-HA* and pROK empty vector transformant lines for tolerance to cadmium salts and the GS-conjugable xenobiotic 1-chloro-2,4-dinitrobenzene (CDNB). CDNB has three advantages for studies of this type: (i) It is an established plant toxin (Li *et al.*, 1995, *Plant Physiol.* 109:117-185); (ii) The kinetics of transport of its glutathionated derivative, DNP-GS, as well characterized for YCF1 (Li *et al.*, 1996, *J. Biol. Chem.* 271:6509-6517) and the endogenous GS-X pump (Li *et al.*, 1995, *Plant. Physiol.* 107:1257-1268; Li *et al.*, 1995, *Plant Physiol.* 109:117-185). (iii) DNP-GS is the only known immediate metabolite of CDNB *in vivo* (Li *et al.*, 1995, *Plant Physiol.* 109:117-185).

Methods similar to those described by Howden *et al.* (Howden *et al.*,1992, *Plant Physiol.* 99:100-107; Howden *et al.*, 1995, *Plant Physiol.* 107:1059-1066; Howden *et al.*, 1995, *Plant Physiol.* 107:1067-1073) were applied to the initial characterization of the transformants. T2 seeds were first sown in rows on Cd^{2+}-free and CDNB-free medium in Petri dishes standing on edge so that the roots grew vertically down the surface of the agar. Three to 4 days after germination, the seeds were transferred, again in rows, to media containing a range of $CdSO_4$ or CDNB concentrations. After rotating the Petri dishes though 180° and allowing growth for another 24-48 hours, the seedlings were scored for hook length. The results of this study are shown in Figure 28. It is evident from the data presented therein that transgenic *Arabiposis* plants comprising *YCF1* acquire increased resistance to cadmium salts and the organic cytotoxin, CDNB.

The disclosures of each and every patent, patent application and publication cited herein are hereby incorporated herein by reference in their entirety.

While this invention has been disclosed with reference to specific embodiments, it is apparent that other embodiments and variations of this invention may be devised by others skilled in the art without departing from the true spirit and scope of the invention. The appended claims are intended to be construed to include all

5   such embodiments and equivalent variations.

SEQUENCE LISTING

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   (ii) TITLE OF INVENTION: GLUTATHIONE-S-CONJUGATE TRANSPORT IN
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  (iii) NUMBER OF SEQUENCES: 20

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    (v) COMPUTER READABLE FORM:
        (A) MEDIUM TYPE: Floppy disk
        (B) COMPUTER: IBM PC compatible
        (C) OPERATING SYSTEM: PC-DOS/MS-DOS
        (D) SOFTWARE: PatentIn Release #1.0, Version #1.30

   (vi) CURRENT APPLICATION DATA:
        (A) APPLICATION NUMBER: Not yet assigned
        (B) FILING DATE: 18-NOV-1997
        (C) CLASSIFICATION:

  (vii) PRIOR APPLICATION DATA:
        (A) APPLICATION NUMBER: US 60/031,040
        (B) FILING DATE: 18-NOV-1996

 (viii) PRIOR APPLICATION DATA:
        (A) APPLICATION NUMBER: US 60/061,328
        (B) FILING DATE: 08-OCT-1997

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(2) INFORMATION FOR SEQ ID NO:1:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 5232 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: double
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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(2) INFORMATION FOR SEQ ID NO:2:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 9936 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: double
        (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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ATCTCTTAAA ATGTATATTA CTGAATTGGC TATTTGATAT TTTCTGTATC CTTTTTAGTT     1800

TCCAGCATTC CTGGGATAAG GAACTACAAA AGCCGCAACC GTGGCTGTTG AGAGCATTGA     1860

ACAATAGCCT GGGAGGAAGG TAGATAGATT TTCTCACCTT ATCGTGCTGT GTTCTCATCT     1920

CTTTTGAGTT TTGAGTATGA TTAGATAGTG CTGGATTTCA CTGTGATGTG CAGATGTTTA     1980

AGTGATCTCT TGAAAGAACC ATCAGGTTTT TAGAATGTGT AGGAAGCAAG ATCAGAATAT     2040

TTCTACTTAT TTAATGTTAG TTGTTTGCTA TAGCAGCTTA ACACATTTCC ATCTTATCAT     2100

AGGCAATCAT GCTTGCTTTC GTACTCTTAT AAATTTAAGA CATAGGGGAT ACAACTTTTA     2160

CTGTAGATTG GTTAAATATG TTTTTTTTTC TTGGTTCATA TTGCTTAAGC ATTATTTCGT     2220

TTGTTAACTA CATGTCGTAT GGGGATCTAA TTTTTTGAAT TTTGTAGGTT TTGGTGGGGA     2280

GGATTTTGGA AGGTATTTTC GTCTACCTCT TTCTCTTTTA TTCGTGCTTC CAGAGTCTTT     2340

CCTCTCTTTT ATTCATATGA TCACAGGTTC TGCGTCATGT TGGATAACCT TCTGTCACGT     2400

GGAAGTCATT TATAATTTAC ATGGTGTTAC AGATTATTAG AAGGAACTAG TGGGTTCTTA     2460

GTTTTTCTTT ATCAATTCAT TGTACTTGAA CATATTTATT TACATTTGTA TGCACAGATC     2520

GGGAATGATT GCTCACAGTT TGTGGGACCT CTTTTACTGA ATCAACTCTT AAAGGTTTGT     2580

TCTTTTCTTG GCAGATTCGG AAACCTATTA TTGGTTCAAT ATTCTTATCT GACAATATCT     2640

CTCATTTTGG ATGTCAAACT ATATACAGTC AATGCAAGAG GATGCGCCAG CTTGGATGGG     2700

TTACATCTAT GCGTTCTCAA TCTTTGGTGG AGTGGTATGA AATGAAGTCC TCTTTCTCTC     2760

TCTCTCTCTG TCTATTTGGA CTCTCTTCTA TCAACTTGTG AAACTGACAC TTGTTATACT     2820
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TCTGTATGTT TGGTCTAAGG TTCTTCTAAA CTGATTATAA TAGCAACACT AGATGTCCCC    2880

TAATGCCACT TTTTGATTTT GTTGCTCTTG GATTTTTTGC GTCTGTTAGA TAGGTTCTGA    2940

CTTTATCTAG TGTAGGGTGA TACTTAAAGC TACAAACTCA TCGAGTGACT GATGTTGATG    3000

ACAACGTTTC TAGGTGTTCG GGGTGCTATG TGAAGCTCAA TATTTCCAGA ATGTCATGCG    3060

TGTTGGTTAC CGACTGAGAT CTGCTCTGGT AAATTTTAAA TTTGCTACCC TGACGTTCTT    3120

CCTTTGCCAT ATGTTTTTGG TGCAGATATG TTTGCTGATA GCATGATTCC CAGTATCTTG    3180

TATAGGAATA AGTATATCAA CATGGTTTCT TTATCCTCTA TATATGATGC ATAAATAAGC    3240

CTTGTGCCAA AAGTTTAGGA ATAAGTTTGT GTTGCTTCAG ATGATTGAGT ATGCTGTTTT    3300

TATTTCTGGA AATTTCCACC ATTTTCAGAT CCTTTCACTA GAGAAATACA AATTTAGCTG    3360

TATTTCCTGA TTCAGTTCAT CGTTTTCTGC GTTTGTAGTG GAGTGAAATT AGCTTGTACG    3420

AAATGGAAGA TATTTTGAAC ACAGATGATT TTTAAAATTG GTCTTCCTGT TGATGACTGT    3480

TTTTTTTTTA GATTGCTGCT GTGTTCCGCA AATCGTTGAG GTTAACTAAT GAAGGTCGTA    3540

GAAAGTTTCA AACAGGAAAG ATAACCAACT TAATGACGAC TGATGCCGAA TCTCTTCAGG    3600

TGAGTATCCC TTTCATATTT TCGAATTCAA GTTTGCATGT TTCTCTATAT CATAGTTGCA    3660

GGGCTGTTAA CATCCGGATC TTGAATATTT ATTTTTGTCC GCAGCTGGTA TTGAGTGGGT    3720

TACAGTTACT TTTTATGTTC GGTAATAGAA GTTGGATTTA CTTAGAAATG ATTTCCAGCA    3780

TACTGATCTA CTGAATCTGT TTGTTAGGTC TAAGATTGGC TATGAATAGT GATTGCATTT    3840

TCATTTCTAG CTAGCACTTT GTTATCATTG AATTTTTCTT TCTTCTTTTT TATTTTGTTT    3900

CTTATGCCAA CTTAAACTGT GTCTTGTTTA ATGTTTTCGT CTTAACTGTG TCTGGTATCA    3960

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AAATATGCCA ATCACTTCAT ACCATGTGGT CGGCTCCATT TCGTATAATT ATAGCACTGA    4080

TTCTCCTCTA TCAGCAATTG GGTGTTGCCT CGCTCATTGG TGCATTGTTG TTGGTCCTTA    4140

TGTTCCCTTT ACAGGTACAT GACTTCTAAA TTTCCTCATT TTTTTTCCTT TGTAGCTTAT    4200

TTTTCTCTAT ACTGTTCGCT TGTTCATTCG TACTCCTAAA GGCTACTTCT TCTTCGTCTC    4260

CTGAACTTGT TCTCTGTTTT CTTAAAACAG ACTGTTATTA TAAGCAAAAT GCAGAAGCTG    4320
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ACAAAGGAAG GTCTGCAGCG TACTGACAAG AGAATTGGCC TTATGAATGA AGTTTTAGCT        4380

GCAATGGATA CAGTAAAGTA AGAAATTCTA GAACCAATTT TGTTAACATA GTTATTAATT        4440

TGCAGGAAAC TTGTACTAAA CCAAAATGCT ACAGGTGTTA TGCTTGGGAA AACAGTTTCC        4500

AGTCCAAGGT CCAAACTGTC GTGATGATGA ATTATCTTGG TTCCGGAAAT CACAGCTCCT        4560

GGGAGCGGTA TGACTACAGC GTAGTTACTT TTGTTTTTCC TCTAATTATT GTATATTTCT        4620

AACTCTTGCT TGGTCTTGTC TTGTTTTGCA GTTGAATATG TTCATACTGA ATAGCATTCC        4680

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TGCAAGAGCA TTTACGTCAC TCTCTCTCTT TGCTGTGCTT CGTTCCCTC TCTTCATGCT         4800

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TAGAACTGTT AGTGCTTGTA GTAACTGTCG TTTGGTTATC AAATCCATGA CTTATATTTC        4920

GAATTTACAT GCTGGAGGGT ATCCTTGCTG GTGCCAGAAA CAGATGCCGA TGCTGACTAG        4980

TTTTCACTTG TAGGTGGTAA ATGCTAATGT ATCCTTAAAA CGTCTTGAGG AGGTATTGGC        5040

GACAGAAGAA AGAATTCTCT TACCAAATCC TCCCATTGAA CCTGGAGAGC CAGCCATCTC        5100

AATAAGAAAT GGATATTTCT CTTGGGATTC TAAGGTGTCG CTTGGCTATT CTATACCATG        5160

TTCCTTCTTT CGCTTCTCTC ATTACCTTTA TCCATAGAAA GTACAAAAAT CGAGCTAACC        5220

CTATGTATCT ACAGGGGGAT AGGCCGACGT TGTCAAATAT CAACTTGGAT GTACCTCTTG        5280

GCAGCCTAGT TGCTGTGGTT GGTAGTACAG GCGAAGGAAA AACCTCTCTA ATATCTGCTA        5340

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TTTTATAGTA GAACAATATG TGTTCTCAAA TGCGTCAGTT ACTTCACCAA CTTAGTGGAA        5580

ACCTTCTTCA ATATTTGATT CTCTAAGCTA TTTTGAACAG AAGACTGATA TGCATTTTCT        5640

TATAAAAATT TGTAGGTACG CGACAATATA CTGTTTGGTT CTCCTTTCGA CCGTGAAAAG        5700

TATGAAAGGG CCATTGATGT GACTTCACTG AAGCATGACC TAGAGTTACT GCCTGTAAGT        5760

TTTGAGGAGA GCTTCGTGGA GTTGATAACA AGGATTTGTC TTGCCTGTTC TCGTGTTGCT        5820
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AAGTTTGTTT CAACCTCTTT CTCTTGCTTA ATAGGGTGGT GATCTCACGG AGATTGGAGA   5880

AAGAGGTGTT AATATCAGTG GAGGACAGAA GCAGAGGGTT TCCATGGCTA GGGCCGTTTA   5940

CTCAAATTCA GATGTGTACA TCTTTGATGA CCCGTTAAGT GCCCTTGATG CTCATGTTGG   6000

TCAACAGGTA CTAACTCATT GATTCTCTTT GATAAGGCTA GTCTATTTCA TTTTTGAATT   6060

TATCTAACAT TTTTGTGTCT GGTCATTATG GGAATACTGT CAGTCTGATT TCTAGGAATA   6120

TTGTTTCAGG TTTTTGAAAA ATGCATAAAA AGAGAACTGG GGCAGAAAAC GAGAGTTCTT   6180

GTTACAAACC AGCTCCACTT CCTATCACAA GTGGACAGAA TTGTACTTGT GCATGAAGGC   6240

ACAGTGAAAG AGGAAGGAAC ATATGAAGAG CTATCCAGTA ATGGGCCTTT GTTCCAGAGG   6300

GTAATGGAAA ATGCAGGGAA GGTGGAAGAA TATTCAGAAG AAAATGGAGA AGCTGAGGCA   6360

GACCAAACAG CGGAACAACC AGTTGCGAAT GGGAACACAA ATGGTCTTCA AATGGATGGA   6420

AGTGACGATA AAAAATCCAA AGAAGGAAAT AAAAAAGGAG GGAAATCTGT CCTCATCAAG   6480

CAAGAAGAAC GTGAAACCGG AGTTGTAAGT TGGAGAGTCC TGAAGAGGTA ACTTGAACAT   6540

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CACTTGGAGG GGCATGGGTA GTGATGATGC TCCTTTTATG TTACGTCTTA ACAGAAGTAT   6780

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CTTCTTTCAA CTGTAGTGTT GATTGGCATT GTAAGCACTT TGTCCTTGTG GGCCATCATG   7380

CCCCTCCTGG TCTTGTTTTA TGGAGCTTAT CTTTATTATC AGGTAATGTA CCTTCTGACC   7440

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CACAGTTTGG AGAGGCATTG AATGGCTTAT CAACTATCCG TGCTTACAAA GCATATGATC   7740

GTATGGCTGA TATCAACGGA AGATCAATGG ATAATAACAT CAGATTCACT CTTGTCAACA   7800

TGGGTGCCAA TCGGTGGCTT GGAATCCGTT TAGAAACTCT GGGTGGTCTT ATGATATGGC   7860

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GAAGGATCTT AATCGATGAT TGTGACGTTG GAAAGTTTGG ACTGATGGAC CTACGTAAAG   8340

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ATCCATTTGG TGAACACAAT GATGCTGATC TTTGGGAATC TCTAGAGAGG GCACACTTGA   8460

AGGATACCAT CCGCAGAAAT CCTCTTGGTC TTGATGCTGA GGTATTCAGT TGCTGCCTAT   8520

ATTGATATGA AGTCTCATTT TTTAAGTGGT AATAACTGAT TTTCAATCTT TGTTCAGGTC   8580

TCTGAGGCAG GAGAGAATTT CAGCGTGGGA CAGAGGCAAT TGTTGAGTCT TTCACGTGCG   8640

CTGTTACGGA GATCTAAGAT ACTCGTCCTT GATGAAGCAA CTGCTGCTGT AGATGTTAGA   8700

ACCGATGCCC TCATTCAGAA GACTATCCGA GAAGAATTCA AGTCATGCAC GATGCTCATT   8760

ATCGCTCACC GTCTCAATAC CATCATTGAC TGTGACAAAA TTCTCGTGCT TGATTCTGGA   8820

AGAGTATGAT TTTAAACACT CTCTCTCTTT CAATCTCACA CTCTCCTTGT TTCTCAGCTA   8880

ACCTGTTCTA TTCCAATTTG TTAACTCAGG TTCAAGAATT CAGTTCACCG GAGAACCTTC   8940

TTTCAAATGA AGGAAGCTCT TTCTCCAAGA TGGTTCAAAG CACTGGAGCT GCAAATGCTG   9000

AGTACTTGCG TAGTTTAGTA CTCGACAACA AGCGTGCCAA AGATGACTCA CACCACTTAC   9060

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CTGCGAGTCT TACTTCGTCG CACAACGATC TTCAAAGCCT TGAAATTGAA GATGACAGCA   9180

GCATTTTGAA GAGAACAAAC GATGCAGTTG TGACTCTGCG CAGTGTTCTC GAGGGGAAAC   9240

ACGACAAAGA GATTGCAGAG TCGCTTGAGG AACATAATAT CTCTAGAGAG GGATGGTTGT   9300

CATCACTCTA TAGAATGGTA GAAGGTAAAC CAAATATGCA TCTCTACAAA TGCTTATGCA   9360

AAATCTTAAT CACCACACTG AAACATTAAA GTCAAATCGT GCTCTTATAT TGCAAGCCTG   9420

CTTTCCGCTG TCTACGTTTC AGGGCTTGCA GTGATGAGCA GATTGGCAAG GAACCGAATG   9480

CAACAACCGG ATTACAATTT CGAAGGAAAT ACATTTGACT GGGACAACGT CGAGATGTAG   9540

ATAAGTTCAT GTTAAACTAG GAATCATTGT CTCTTCCGTA AGAAACATAT ATTTATCTTA   9600

ACCAAAATTA TTAGTTTGGT TTCCATTTCA TAAACTTAAT TTTCACCTGC AAAGAAAATC   9660

AAACCCTGTT GTGTTCTTCG TGATAAGTAG AGAAATTACT TGAGTATCCT TCTAACTCAT   9720

AAATGGGATC TCATGATTCA TGAACAAGCA GCAACACAAT AATACCCTTT TCAGATTTTG   9780

GAGCTGGACA AAGTTGTTAA GTTGAGTTTC TCTTACAGTC ATTCATATAC AAAAACCTCT   9840

TCGACTGAAG CACCAAGAAA GAAACAAACA TCAAAGGGA ATGAGGTCTT TTCTTAGGGC   9900

TGAGATCATC GGAATGTGGG AGTGCGGAAC ACGACC   9936

(2) INFORMATION FOR SEQ ID NO:3:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 1621 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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Met Gly Phe Glu Phe Ile Glu Trp Tyr Cys Lys Pro Val Pro Asn Gly
1                5                   10                  15

Val Trp Thr Lys Thr Val Ala Asn Ala Phe Gly Ala Tyr Thr Pro Cys
            20              25                  30

Ala Thr Asp Ser Phe Val Leu Gly Ile Ser Gln Leu Val Leu Leu Val
        35                  40                  45

Leu Cys Leu Tyr Arg Ile Trp Leu Ala Leu Lys Asp His Lys Val Glu
    50                  55                  60

Arg Phe Cys Leu Arg Ser Arg Leu Tyr Asn Tyr Phe Leu Ala Leu Leu
65                  70                  75                      80

Ala Ala Tyr Ala Thr Ala Glu Pro Leu Phe Arg Leu Ile Met Gly Ile
                85                  90                  95

Ser Val Leu Asp Phe Asp Gly Pro Gly Leu Pro Pro Phe Glu Ala Phe
            100                 105                 110

Gly Leu Gly Val Lys Ala Phe Ala Trp Gly Ala Val Met Val Met Ile
        115                 120                 125

Leu Met Glu Thr Lys Ile Tyr Ile Arg Glu Leu Arg Trp Tyr Val Arg
    130                 135                 140

Phe Ala Val Ile Tyr Ala Leu Val Gly Asp Met Val Leu Leu Asn Leu
145                 150                 155                     160

Val Leu Ser Val Lys Glu Tyr Tyr Ser Ser Tyr Val Leu Tyr Leu Tyr
                165                 170                 175

Thr Ser Glu Val Gly Ala Gln Val Leu Phe Gly Ile Leu Leu Phe Met
            180                 185                 190

His Leu Pro Asn Leu Asp Thr Tyr Pro Gly Tyr Met Pro Val Arg Ser
        195                 200                 205

Glu Thr Val Asp Asp Tyr Glu Tyr Glu Glu Ile Ser Asp Gly Gln Gln
    210                 215                 220

Ile Cys Pro Glu Lys His Pro Asn Ile Phe Asp Lys Ile Phe Phe Ser
225                 230                 235                     240

Trp Met Asn Pro Leu Met Thr Leu Gly Ser Lys Arg Pro Leu Thr Glu
                245                 250                 255
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Lys Asp Val Trp Tyr Leu Asp Thr Trp Asp Gln Thr Glu Thr Leu Phe
        260   265   270

Thr Ser Phe Gln His Ser Trp Asp Lys Glu Leu Gln Lys Pro Gln Pro
        275   280   285

Trp Leu Leu Arg Ala Leu Asn Asn Ser Leu Gly Gly Arg Phe Trp Trp
        290   295   300

Gly Gly Phe Trp Lys Ile Gly Asn Asp Cys Ser Gln Phe Val Gly Pro
305   310   315   320

Leu Leu Leu Asn Gln Leu Leu Lys Ser Met Gln Glu Asp Ala Pro Ala
                325   330   335

Trp Met Gly Tyr Ile Tyr Ala Phe Ser Ile Phe Gly Gly Val Val Phe
                340   345   350

Gly Val Leu Cys Glu Ala Gln Tyr Phe Gln Asn Val Met Arg Val Gly
        355   360   365

Tyr Arg Leu Arg Ser Ala Leu Ile Ala Ala Val Phe Arg Lys Ser Leu
        370   375   380

Arg Leu Thr Asn Glu Gly Arg Arg Lys Phe Gln Thr Gly Lys Ile Thr
385   390   395   400

Asn Leu Met Thr Thr Asp Ala Glu Ser Leu Gln Gln Ile Cys Gln Ser
                405   410   415

Leu His Thr Met Trp Ser Ala Pro Phe Arg Ile Ile Ile Ala Leu Ile
                420   425   430

Leu Leu Tyr Gln Gln Leu Gly Val Ala Ser Leu Ile Gly Ala Leu Leu
        435   440   445

Leu Val Leu Met Phe Pro Leu Gln Thr Val Ile Ile Ser Lys Met Gln
        450   455   460

Lys Leu Thr Lys Glu Gly Leu Gln Arg Thr Asp Lys Arg Ile Gly Leu
465   470   475   480

Met Asn Glu Val Leu Ala Ala Met Asp Thr Val Lys Cys Tyr Ala Trp
                485   490   495

Glu Asn Ser Phe Gln Ser Lys Val Gln Thr Val Arg Asp Asp Glu Leu
                500   505   510

Ser Trp Phe Arg Lys Ser Gln Leu Leu Gly Ala Leu Asn Met Phe Ile
        515   520   525

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Leu Asn Ser Ile Pro Val Leu Val Thr Ile Val Ser Phe Gly Val Phe
    530   535   540

Thr Leu Leu Gly Gly Asp Leu Thr Pro Ala Arg Ala Phe Thr Ser Leu
545   550   555   560

Ser Leu Phe Ala Val Leu Arg Phe Pro Leu Phe Met Leu Pro Asn Ile
                565   570   575

Ile Thr Gln Val Val Asn Ala Asn Val Ser Leu Lys Arg Leu Glu Glu
            580   585   590

Val Leu Ala Thr Glu Glu Arg Ile Leu Leu Pro Asn Pro Pro Ile Glu
    595   600   605

Pro Gly Glu Pro Ala Ile Ser Ile Arg Asn Gly Tyr Phe Ser Trp Asp
    610   615   620

Ser Lys Gly Asp Arg Pro Thr Leu Ser Asn Ile Asn Leu Asp Val Pro
625   630   635   640

Leu Gly Ser Leu Val Ala Val Val Gly Ser Thr Gly Glu Gly Lys Thr
                645   650   655

Ser Leu Ile Ser Ala Ile Leu Gly Glu Leu Pro Ala Thr Ser Asp Ala
            660   665   670

Ile Val Thr Leu Arg Gly Ser Val Ala Tyr Val Pro Gln Val Ser Trp
    675   680   685

Ile Phe Asn Ala Thr Val Arg Asp Asn Ile Leu Phe Gly Ser Pro Phe
    690   695   700

Asp Arg Glu Lys Tyr Glu Arg Ala Ile Asp Val Thr Ser Leu Lys His
705   710   715   720

Asp Leu Glu Leu Leu Pro Gly Gly Asp Leu Thr Glu Ile Gly Glu Arg
                725   730   735

Gly Val Asn Ile Ser Gly Gly Gln Lys Gln Arg Val Ser Met Ala Arg
                740   745   750

Ala Val Tyr Ser Asn Ser Asp Val Tyr Ile Phe Asp Asp Pro Leu Ser
            755   760   765

Ala Leu Asp Ala His Val Gly Gln Gln Val Phe Glu Lys Cys Ile Lys
770   775   780

Arg Glu Leu Gly Gln Lys Thr Arg Val Leu Val Thr Asn Gln Leu His
785   790   795   800

Phe Leu Ser Gln Val Asp Arg Ile Val Leu Val His Glu Gly Thr Val
805   810   815

Lys Glu Glu Gly Thr Tyr Glu Glu Leu Ser Ser Asn Gly Pro Leu Phe
820   825   830

Gln Arg Leu Met Glu Asn Ala Gly Lys Val Glu Glu Tyr Ser Glu Glu
835   840   845

Asn Gly Glu Ala Glu Ala Asp Gln Thr Ala Glu Gln Pro Val Ala Asn
850   855   860

Gly Asn Thr Asn Gly Leu Gln Met Asp Gly Ser Asp Asp Lys Lys Ser
865   870   875   880

Lys Glu Gly Asn Lys Lys Gly Gly Lys Ser Val Leu Ile Lys Gln Glu
885   890   895

Glu Arg Glu Thr Gly Val Val Ser Trp Arg Val Leu Lys Arg Tyr Gln
900   905   910

Asp Ala Leu Gly Gly Ala Trp Val Val Met Met Leu Leu Leu Cys Tyr
915   920   925

Val Leu Thr Glu Val Phe Arg Val Thr Ser Ser Thr Trp Leu Ser Glu
930   935   940

Trp Thr Asp Ala Gly Thr Pro Lys Ser His Gly Pro Leu Phe Tyr Asn
945   950   955   960

Leu Ile Tyr Ala Leu Leu Ser Phe Gly Gln Val Leu Val Thr Leu Thr
965   970   975

Asn Ser Tyr Trp Leu Ile Met Ser Ser Leu Tyr Ala Ala Lys Lys Leu
980   985   990

His Asp Asn Met Leu His Ser Ile Leu Arg Ala Pro Met Ser Phe Phe
995   1000   1005

His Thr Asn Pro Leu Gly Arg Ile Ile Asn Arg Phe Ala Lys Asp Leu
1010   1015   1020

Gly Asp Ile Asp Arg Thr Val Ala Val Phe Val Asn Met Phe Met Gly
1025   1030   1035   1040

Gln Val Ser Gln Leu Leu Ser Thr Val Val Leu Ile Gly Ile Val Ser
1045   1050   1055

Thr Leu Ser Leu Trp Ala Ile Met Pro Leu Leu Val Leu Phe Tyr Gly
1060   1065   1070

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Ala Tyr Leu Tyr Tyr Gln Asn Thr Ala Arg Glu Val Lys Arg Met Asp
        1075                1080                1085

Ser Ile Ser Arg Ser Pro Val Tyr Ala Gln Phe Gly Glu Ala Leu Asn
        1090                1095                1100

Gly Leu Ser Thr Ile Arg Ala Tyr Lys Ala Tyr Asp Arg Met Ala Asp
1105                1110                1115                1120

Ile Asn Gly Arg Ser Met Asp Asn Asn Ile Arg Phe Thr Leu Val Asn
                1125                1130                1135

Met Gly Ala Asn Arg Trp Leu Gly Ile Arg Leu Glu Thr Leu Gly Gly
            1140                1145                1150

Leu Met Ile Trp Leu Thr Ala Ser Phe Ala Val Met Gln Asn Gly Arg
        1155                1160                1165

Ala Glu Asn Gln Gln Ala Phe Ala Ser Thr Met Gly Leu Leu Leu Ser
        1170                1175                1180

Tyr Ala Leu Asn Ile Thr Ser Leu Leu Thr Gly Val Leu Arg Leu Ala
1185                1190                1195                1200

Ser Leu Ala Glu Asn Ser Leu Asn Ala Val Glu Arg Val Gly Asn Tyr
                1205                1210                1215

Ile Glu Ile Pro Pro Glu Ala Pro Pro Val Ile Glu Asn Asn Arg Pro
                1220                1225                1230

Pro Pro Gly Trp Pro Ser Ser Gly Ser Ile Lys Phe Glu Asp Val Val
            1235                1240                1245

Leu Arg Tyr Arg Pro Gln Leu Pro Pro Val Leu His Gly Val Ser Phe
    1250                1255                1260

Phe Ile His Pro Thr Asp Lys Val Gly Ile Val Gly Arg Thr Gly Ala
1265                1270                1275                1280

Gly Lys Ser Ser Leu Leu Asn Ala Leu Phe Arg Ile Val Glu Val Glu
                1285                1290                1295

Glu Gly Arg Ile Leu Ile Asp Asp Cys Asp Val Gly Lys Phe Gly Leu
            1300                1305                1310

Met Asp Leu Arg Lys Val Leu Gly Ile Ile Pro Gln Ser Pro Val Leu
        1315                1320                1325

Phe Ser Gly Thr Val Arg Phe Asn Leu Asp Pro Phe Gly Glu His Asn
1330                1335                1340
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Asp Ala Asp Leu Trp Glu Ser Leu Glu Arg Ala His Leu Lys Asp Thr
1345   1350   1355   1360

Ile Arg Arg Asn Pro Leu Gly Leu Asp Ala Glu Val Ser Glu Ala Gly
                1365   1370   1375

Glu Asn Phe Ser Val Gly Gln Arg Gln Leu Leu Ser Leu Ser Arg Ala
                1380   1385   1390

Leu Leu Arg Arg Ser Lys Ile Leu Val Leu Asp Glu Ala Thr Ala Ala
                1395   1400   1405

Val Asp Val Arg Thr Asp Ala Leu Ile Gln Lys Thr Ile Arg Glu Glu
                1410   1415   1420

Phe Lys Ser Cys Thr Met Leu Ile Ile Ala His Arg Leu Asn Thr Ile
1425   1430   1435   1440

Ile Asp Cys Asp Lys Ile Leu Val Leu Asp Ser Gly Arg Val Gln Glu
                1445   1450   1455

Phe Ser Ser Pro Glu Asn Leu Leu Ser Asn Glu Gly Ser Ser Phe Ser
                1460   1465   1470

Lys Met Val Gln Ser Thr Gly Ala Ala Asn Ala Glu Tyr Leu Arg Ser
                1475   1480   1485

Leu Val Leu Asp Asn Lys Arg Ala Lys Asp Asp Ser His His Leu Gln
                1490   1495   1500

Gly Gln Arg Lys Trp Ala Ser Ser Arg Trp Ala Ala Ala Ala Gln Phe
1505   1510   1515   1520

Ala Leu Ala Ala Ser Leu Thr Ser Ser His Asn Asp Leu Gln Ser Leu
                1525   1530   1535

Glu Ile Glu Asp Asp Ser Ser Ile Leu Lys Arg Thr Asn Asp Ala Val
                1540   1545   1550

Val Thr Leu Arg Ser Val Leu Glu Gly Lys His Asp Lys Glu Ala Glu
                1555   1560   1565

Ser Leu Glu Glu His Asn Ile Ser Arg Glu Gly Trp Leu Ser Ser Leu
                1570   1575   1580

Tyr Arg Met Val Glu Gly Leu Ala Val Met Ser Arg Leu Ala Arg Asn
1585   1590   1595   1600

Arg Met Gln Gln Pro Asp Tyr Asn Phe Glu Gly Asn Thr Phe Asp Trp
                1605   1610   1615

- 110 -

Asp Asn Val Glu Met
            1620

(2) INFORMATION FOR SEQ ID NO:4:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 5175 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: double
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GAATTCGCGG CCGCCGGCGA ATTTGCACTC TTTACCTCTC TTTGACTCCG TGAGATTCGA   60

GGATTGTTAG TTTCTTGTGA TGTGTAGTCT TTGAAGCAGG GGATTTTTAT TGTATTGAGG   120

AAGAAGATGG GGTTTGAGCC GTTGGATTGG TATTGCAAGC CGGTGCCGAA TGGTGTGTGG   180

ACTAAAACTG TGGATTATGC GTTTGGTGCA TACACGCCTT GTGCTATTGA CTCTTTTGTG   240

CTTGGTATCT CTCATCTGGT TCTGTTGATT CTGTGTCTTT ATCGCTTGTG GCTCATCACG   300

AAGGATCACA AAGTGGATAA GTTCTGCTTG AGGTCTAAAT GGTTTAGCTA TTTTCTGGCT   360

CTTTTGGCTG CTTATGCTAC TGCGGAGCCT TTGTTTAGAT TGGTCATGAG GATCTCTGTT   420

TTGGATTTGG ATGGAGCTGG GTTTCCTCCC TATGAGGCGT TTATGTTGGT CCTTGAGGCT   480

TTTGCTTGGG GTTCTGCTTT GGTCATGACT GTTGTGGAAA CTAAAACGTA TATCCATGAA   540

CTCCGTTGGT ATGTCAGATT CGCTGTCATT TATGCTCTTG TGGGAGACAT GGTGTTGTTA   600

AATCTTGTTC TCTCTGTTAA GGAGTACTAT GGCAGTTTTA AACTGTATCT TTACATAAGC   660

GAGGTGGCAG TTCAGGTTGC ATTTGGAACC CTCTTGTTTG TGTATTCCCC TAATTTGGAC   720

CCTTACCCTG GTTACACACC AGTTGGGACT GAAAATTCCG AGGATTACGA GTATGAAGAG   780

CTTCCTGGAG GAGAAAATAT ATGTCCTGAG AGGCATGCAA ATTTATTTGA CAGTATCTTC   840

TTCTCATGGT TGAACCCATT GATGACTCTG GGATCAAAAC GACCTCTCAC CGAGAAGGAT   900

GTATGGCATC TGGACACTTG GGATAAAACT GAAACTCTTA TGAGGAGCTT CCAGAAGTCC   960

TGGGATAAGG AACTAGAAAA GCCCAAACCG TGGCTTTTGA GAGCACTGAA CAACAGCCTT   1020

GGGGGAAGGT TTTGGTGGGG TGGCTTTTGG AAGATTGGGA ATGACTGTTC ACAGTTCGTG   1080

GGGCCTCTTC TACTGAATGA GCTCTTAAAG TCAATGCAAC TTAATGAACC AGCGTGGATA   1140

GGTTACATCT ATGCAATCTC AATCTTTGTT GGAGTGGTAT TGGGGGTTTT ATGTGAAGCT   1200

CAGTATTTCC AAAATGTGAT GCGTGTTGGT TACCGGCTTA GGTCTGCACT GATTGCTGCT   1260

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TATCTGCTAT GCTTGGGGAA CTTCCTGCAA GATCTGATGC GACTGTTACT CTTAGAGGAT   2160

CAGTCGCTTA TGTTCCACAA GTTTCATGGA TCTTTAACGC AACAGTACGT GACAATATAT   2220

TGTTTGGGGC TCCTTTTGAC CAAGAAAAAT ATGAAAGGGT GATTGATGTG ACAGCACTCC   2280

AGCATGACCT TGAGTTACTG CCTGGAGGTG ACCTCACGGA GATCGGAGAA AGGGGTGTTA   2340

ACATCAGTGG GGGACAAAAG CAGAGGGTTT CTATGGCTAG GGCCGTTTAC TCAAATTCAG   2400

ACGTGTGCAT CTTAGATGAA CCATTGAGTG CCCTTGATGC GCATGTTGGT CAGCAGGTTT   2460

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AAGGAACATA TGAAGAATTA TGCCATAGTG GCCCGTTGTT CCCGAGGTTA ATGGAAAATG    2640

CAGGGAAGGT TGAAGATTAT TCCGAAGAAA ATGGAGAAGC TGAAGTACAT CAAACATCTG    2700

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CATTGGTCAT TGAAACAACC CGTCCACCTC CCGGATGGCC ATCATCTGGA TCCATAAAAT    3840

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TCTTGATTTC TCCAATGGAT AAGGTGGGAA TTGTTGGGAG GACAGGCGCT GGGAAATCAA    3960
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ACGACGCCGA TCTCTGGGAA TCTCTTGAGA GGGCACACTT GAAAGATACT ATCCGCAGAA    4200

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GACAGTTGTT GAGTCTTGCA CGTGCATTGT TACGAAGATC TAAGATACTT GTTCTTGATG    4320

AAGCAACTGC TGCAGTTGAC GTAAGAACTG ATGTTCTCAT CCAAAAGACC ATCCGAGAAG    4380

AATTCAAGTC ATGCACAATG CTAATCATCG CTCATCGTCT CAATACTATC ATCGACTGTG    4440

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CGAGGAACAG AATGCAACAC CCGGATTACA ATTTAGAAGG GAAATCGTTT GACTGGGACA    4980

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GAACTTTTAT TTGTATTGCT TGGGATACAC GTAACAAAAT GCCCATTAAT CGTGGTGTAA    5100

CTATATAGGC TATGCTTCTT TTGGGAAAAA GAGAGTTTGA TTACAGAGGA TGTGATGATA    5160

ACACAATTGG AATTC                                                     5175
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(2) INFORMATION FOR SEQ ID NO:5:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 10342 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: double
        (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

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TCTCCATGAG AATGTATGCT TTTAAACTTT TTTTTTTTTG TTTTTTGCCT TCGGAGCTAA     180

CTTTGGGGGC TGGTCTCGGT CTCTGTTTTC TCTCCACTAA AAAGATAAAA AGCTTTTGCC     240

ATCTTTTTTT TTTTCTCAAT AATCTATCAC ATCGTTTTTT TTCTTTGTTT TTTTCTCCAT     300

TTGTCTTCAT TGAGTTCATA GCCACATAAT TATTGATTTC TTTTTCTTTT AGTGTTTCTG     360

TTACTGATGC GTTTCATTAT TTATACTTCT CACTTGCAGA TTCGAGGATT GTTAGTTTCT     420

TGTGATGTGT AGTCTTTGAA GCAGGGGATT TTTATTGTAT TGAGGAAGAA GATGGGGTTT     480

GAGCCGTTGG ATTGGTATTG CAAGCCGGTG CCGAATGGTG TGTGGACTAA AACTGTGGAT     540

TATGCGTTTG GTGCATACAC GCCTTGTGCT ATTGACTCTT TTGTGCTTGG TATCTCTCAT     600

CTGGTTCTGT TGATTCTGTG TCTTTATCGC TTGTGGCTCA TCACGAAGGA TCACAAAGTG     660

GATAAGTTCT GCTTGAGGTC TAAATGGTTT AGCTATTTTC TGGCTCTTTT GGCTGCTTAT     720

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TTTGCAGTCT GTATGGTTTA TGCAATTTTG TTTCCCTGGT CTGGCACGAT GAACTTATAT    1140

GCGTCATTTT TTTTTTGTTT TTGGCAGTTT TAAACTGTAT CTTTACATAA GCGAGGTGGC    1200

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CTGTATCTAA GTCACACTGT AAATGATACA ACTTGTTTTT TATAATGACG TTAAGGATGG    1320
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- 115 -

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TCCTGGAGGA GAAAATATAT GTCCTGAGAG GCATGCAAAT TTATTTGACA GTATGTCACT      1500

CTACACTTCT CATTCCCTAC TTTGTTTTTA TAGGTGCATT TTCTATTTTA ATTGTGAGAA      1560

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GCAGAGCTTA AATATTTCCC TGGAATTGTA AATTTTCTTA TGGAGTGCTA ATACGTAGTA      1680

GGTCATTAAA ATTGTTTCCG CAGAGAGTAG TCTATAGTCT CTTCAAAATT TTTTTTTGAC     1740

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TCATCTTTTT GGGCTTTGAC ATGATGTGTG ATTTGTAAAA GGAAGCATTT GGTTGTAATA   2280

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CTTTTTACCC CGTGCACATT GTGCTTGAAC CTATTTAACA CAATGCTTTG TAATTTTTCC 2760

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GAATTATCTT TTGCTACTTA GTTTTTCTTT TTGCTCTGCT TCTCATGTTT TGATGCAATA    3480

CCTGTACTGT TATGTCTGTT GAAAGCTATA GCAGATGCTT ATAGATTGCT TCATTCTGCT    3540

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GTTGCCTCGA TCATTGGTGC ATTGTTTCTT GTCCTTATGT TCCCCATACA GGTTCGTATA    4200

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TCTCATTTGC TTTTCACACC AATGAAAATG ATTCATTTCC TCCGTTTATT TGGTTGAAAC    4320
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TTGTTTGACT CGCTCTATTC ATATTCATAC AAAATGTATA TATATTTTCC GTATTAAGGT    7140

TCGTATTGTA AAGCCATTGT AATAACTTGT GAGGTGTCAC CATGTTCCAG GTACCAGAAT    7200

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TTTCGGGTTT CAAGCATCAC TTGGTTGAGT GAGTGGACTG ATTCAGGAAC CCCAAAGACT    7320
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GGTGGCTGGG AATCCGTTTG GAAGTTTTGG GAGGTCTCAT GGTTTGGTGG ACTGCTTCAT    8280

TAGCCGTCAT GCAGAACGGA AAGGCAGCGA ACCAACAAGC ATATGCATCT ACGATGGGTT    8340

TGCTTCTCAG TTATGCGTTA AGCATTACCA GCTCTTTAAC AGCTGTACTG AGACTCGCGA    8400

GTCTAGCTGA GAATAGTTTA AACTCGGTTG AGCGTGTTGG AAATTATATC GAGATACCAT    8460

CAGAGGCTCC ATTGGTCATT GAAACAACC GTCCACCTCC CGGATGGCCA TCATCTGGAT    8520

CCATAAAATT TGAGGATGTT GTTCTTCGTT ACCGCCCTGA GTTACCTCCT GTTCTTCATG    8580

GAGTTTCGTT CTTGATTTCT CCAATGGATA AGGTGGGAAT TGTTGGGAGG ACAGGCGCTG    8640

GGAAATCAAG CCTCTTAAAT GCCTTATTCA GGATTGTGGA GCTGGAAAAA GGAAGGATTT    8700

TAATTGATGA ATGCGACATT GGAAGATTTG GACTGATGGA CCTACGTAAA GTGGTCGGAA    8760

TTATACCGCA AGCGCCAGTT CTTTTCTCAG GTACCGTGAG ATTCAATCTT GACCCATTTA    8820
```

GTGAACACAA CGACGCCGAT CTCTGGGAAT CTCTTGAGAG GGCACACTTG AAAGATACTA   8880

TCCGCAGAAA TCCTCTTGGT CTTGATGCTG AGGTACTTAA TTAAATATTT CCATTTGGGA   8940

AAGTCTCATG TATTCAGTAA TAATAACTCA GTCTTTTTGG TCAGGTAACT GAGGCAGGAG   9000

AGAATTTCAG TGTTGGACAG AGACAGTTGT TGAGTCTTGC ACGTGCATTG TTACGAAGAT   9060

CTAAGATACT TGTTCTTGAT GAAGCAACTG CTGCAGTTGA CGTAAGAACT GATGTTCTCA   9120

TCCAAAAGAC CATCCGAGAA GAATTCAAGT CATGCACAAT GCTAATCATC GCTCATCGTC   9180

TCAATACTAT CATCGACTGT GACAAAGTTC TTGTCCTTGA TTCTGGAAAA GTACGTATAC   9240

AAAATATTCG ACCACTACTT GCATCAATTT AATCACTTTT GAGCTAACAT ATATTGAGAT   9300

TCCCAACACC TCAGGTTCAG GAATTCAGTT CACCGGAGAA TCTTCTTTCA AATGGAGAAA   9360

GTTCTTTCTC GAAGATGGTT CAAAGTACAG GAACTGCAAA CGCGGAGTAC TTACGTAGTA   9420

TAACACTAGA GAACAAACGT ACCAGAGAAG CTAACGGTGA TGATTCACAA CCTTTAGAAG   9480

GTCAAAGGAA ATGGCAAGCT TCTTCTCGTT GGGCTGCAGC TGCTCAATTT GCATTGGCTG   9540

TGAGCCTCAC TTCATCTCAC AACGACCTCC AAAGCCTTGA AATCGAAGAT GATAACAGTA   9600

TTTTGAAGAA AACAAAGGAC GCCGTCGTCA CTTTACGCAG TGTCCTTGAA GGGAAACATG   9660

ATAAAGAGAT TGAAGACTCT CTAAACCAAA GTGACATCTC TAGAGAGCGT TGGTGGCCAT   9720

CTCTTTACAA AATGGTCGAA GGTAACGTTA TTCTTAAGAT TTCTGATACG AGTATACGAC   9780

ATAAAGAATT GTTGAAGTTT CTTGATCTAA TAATTTGTGT ATATACTCTC AGGGCTTGCC   9840

GTGATGAGCA GATTGGCGAG GAACAGAATG CAACACCCGG ATTACAATTT AGAAGGGAAA   9900

TCGTTTGACT GGGACAATGT CGAGATGTAA ACGATGAAAG GCTTACACTA ATAGACCTAA   9960

AACTCCCATT TTGATGGAAC TTTTATTTGT ATTGCTTGGG ATACACGTAA CAAAATGCCC   10020

ATTAATCGTG GTGTAACTAT ATAGGCTATG CTTCTTTTGG GAAAAAGAGA GTTTGATTAC   10080

AGAGGATGTG ATGATAACAC AATTGGAATT CAAATTTGCA GCAAAATTTG GGAGAAAAAA   10140

AAAAGTCAAT GAGTGCAACA TGCCAACATG GTTTCAACTT CTGGACATGG ACAACCATTG   10200

GACATAATTT CTCTCACAGG ACCATGTTTT GTCATTGACA TTTTGCACAA AAATGTTCTA   10260

TTAAACATAT ATCTATAAAG AATTTGAACA ATTGTTAAAA AAACACTTAA AATATAAATT   10320

GCAATACAAA TTTCCTTTTT TT   10342

(2) INFORMATION FOR SEQ ID NO:6:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 1622 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: protein

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

```
Met Gly Phe Glu Pro Leu Asp Trp Tyr Cys Lys Pro Val Pro Asn Gly
1               5                   10                  15

Val Trp Thr Lys Thr Val Asp Tyr Ala Phe Gly Ala Tyr Thr Pro Cys
                20                  25                  30

Ala Ile Asp Ser Phe Val Leu Gly Ile Ser His Leu Val Leu Leu Ile
            35                  40                  45

Leu Cys Leu Tyr Arg Leu Trp Leu Ile Thr Lys Asp His Lys Val Asp
    50                  55                  60

Lys Phe Cys Leu Arg Ser Lys Trp Phe Ser Tyr Phe Leu Ala Leu Leu
65                  70                  75                  80

Ala Ala Tyr Ala Thr Ala Glu Pro Leu Phe Arg Leu Val Met Arg Ile
                85                  90                  95

Ser Val Leu Asp Leu Asp Gly Ala Gly Phe Pro Pro Tyr Glu Ala Phe
                100                 105                 110

Met Leu Val Leu Glu Ala Phe Ala Trp Gly Ser Ala Leu Val Met Thr
            115                 120                 125

Val Val Glu Thr Lys Thr Tyr Ile His Glu Leu Arg Trp Tyr Val Arg
    130                 135                 140

Phe Ala Val Ile Tyr Ala Leu Val Gly Asp Met Val Leu Leu Asn Leu
145                 150                 155                 160

Val Leu Ser Val Lys Glu Tyr Tyr Gly Ser Phe Lys Leu Tyr Leu Tyr
                165                 170                 175
```

Ile Ser Glu Val Ala Val Gln Val Ala Phe Gly Thr Leu Leu Phe Val
            180   185   190

Tyr Phe Pro Asn Leu Asp Pro Tyr Pro Gly Tyr Thr Pro Val Gly Thr
        195   200   205

Glu Asn Ser Glu Asp Tyr Glu Tyr Glu Glu Leu Pro Gly Gly Glu Asn
    210   215   220

Ile Cys Pro Glu Arg His Ala Asn Leu Phe Asp Ser Ile Phe Phe Ser
225   230   235   240

Trp Leu Asn Pro Leu Met Thr Leu Gly Ser Lys Arg Pro Leu Thr Glu
            245   250   255

Lys Asp Val Trp His Leu Asp Thr Trp Asp Lys Thr Glu Thr Leu Met
            260   265   270

Arg Ser Phe Gln Lys Ser Trp Asp Lys Glu Leu Glu Lys Pro Lys Pro
        275   280   285

Trp Leu Leu Arg Ala Leu Asn Asn Ser Leu Gly Gly Arg Phe Trp Trp
    290   295   300

Gly Gly Phe Trp Lys Ile Gly Asn Asp Cys Ser Gln Phe Val Gly Pro
305   310   315   320

Leu Leu Leu Asn Glu Leu Leu Lys Ser Met Gln Leu Asn Glu Pro Ala
            325   330   335

Trp Ile Gly Tyr Ile Tyr Ala Ile Ser Ile Phe Val Gly Val Val Leu
            340   345   350

Gly Val Leu Cys Glu Ala Gln Tyr Phe Gln Asn Val Met Arg Val Gly
        355   360   365

Tyr Arg Leu Arg Ser Ala Leu Ile Ala Ala Val Phe Arg Lys Ser Leu
    370   375   380

Arg Leu Thr Asn Glu Gly Arg Lys Lys Phe Gln Thr Gly Lys Ile Thr
385   390   395   400

Asn Leu Met Thr Thr Asp Ala Glu Ser Leu Gln Gln Ile Cys Gln Ser
            405   410   415

Leu His Thr Met Trp Ser Ala Pro Phe Arg Ile Ile Val Ala Leu Val
            420   425   430

Leu Leu Tyr Gln Gln Leu Gly Val Ala Ser Ile Ile Gly Ala Leu Phe
    435   440   445

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Leu Val Leu Met Phe Pro Ile Gln Thr Val Ile Ile Ser Lys Thr Gln
    450                 455             460

Lys Leu Thr Lys Glu Gly Leu Gln Arg Thr Asp Lys Arg Ile Gly Leu
465             470                 475                 480

Met Asn Glu Val Leu Ala Ala Met Asp Thr Val Lys Cys Tyr Ala Trp
                485             490                 495

Glu Asn Ser Phe Gln Ser Lys Val Gln Thr Val Arg Asp Asp Glu Leu
                500             505             510

Ser Trp Phe Arg Lys Ala Gln Leu Leu Ser Ala Phe Asn Met Phe Ile
            515             520             525

Leu Asn Ser Ile Pro Val Leu Val Thr Val Val Ser Phe Gly Val Phe
    530                 535             540

Ser Leu Leu Gly Gly Asp Leu Thr Pro Ala Arg Ala Phe Thr Ser Leu
545             550                 555                 560

Ser Leu Phe Ser Val Leu Arg Phe Pro Leu Phe Met Leu Pro Asn Ile
                565             570             575

Ile Thr Gln Met Val Asn Ala Asn Val Ser Leu Asn Arg Leu Glu Glu
            580             585             590

Val Leu Ser Thr Glu Glu Arg Val Leu Leu Pro Asn Pro Pro Ile Glu
    595                 600             605

Pro Gly Gln Pro Ala Ile Ser Ile Arg Asn Gly Tyr Phe Ser Trp Asp
    610                 615             620

Ser Lys Ala Asp Arg Pro Thr Leu Ser Asn Ile Asn Leu Asp Ile Pro
625             630                 635                 640

Leu Gly Ser Leu Val Ala Val Val Gly Ser Thr Gly Glu Gly Lys Thr
                645             650                 655

Ser Leu Ile Ser Ala Met Leu Gly Glu Leu Pro Ala Arg Ser Asp Ala
            660             665                 670

Thr Val Thr Leu Arg Gly Ser Val Ala Tyr Val Pro Gln Val Ser Trp
    675                 680                 685

Ile Phe Asn Ala Thr Val Arg Asp Asn Ile Leu Phe Gly Ala Pro Phe
    690                 695             700

Asp Gln Glu Lys Tyr Glu Arg Val Ile Asp Val Thr Ala Leu Gln His
705             710                 715                 720
```

Asp Leu Glu Leu Leu Pro Gly Gly Asp Leu Thr Glu Ile Gly Glu Arg
              725   730   735

Gly Val Asn Ile Ser Gly Gly Gln Lys Gln Arg Val Ser Met Ala Arg
              740   745   750

Ala Val Tyr Ser Asn Ser Asp Val Cys Ile Leu Asp Glu Pro Leu Ser
              755   760   765

Ala Leu Asp Ala His Val Gly Gln Gln Val Phe Glu Lys Cys Ile Lys
    770   775   780

Arg Glu Leu Gly Gln Thr Thr Arg Val Leu Val Thr Asn Gln Leu His
785   790   795   800

Phe Leu Ser Gln Val Asp Lys Ile Leu Leu Val His Glu Gly Thr Val
              805   810   815

Lys Glu Glu Gly Thr Tyr Glu Glu Leu Cys His Ser Gly Pro Leu Phe
              820   825   830

Pro Arg Leu Met Glu Asn Ala Gly Lys Val Glu Asp Tyr Ser Glu Glu
              835   840   845

Asn Gly Glu Ala Glu Val His Gln Thr Ser Val Lys Pro Val Glu Asn
    850   855   860

Gly Asn Ala Asn Asn Leu Gln Lys Asp Gly Ile Glu Thr Lys Asn Ser
865   870   875   880

Lys Glu Gly Asn Ser Val Leu Val Lys Arg Glu Glu Arg Glu Thr Gly
              885   890   895

Val Val Ser Trp Lys Val Leu Glu Arg Tyr Gln Asn Ala Leu Gly Gly
              900   905   910

Ala Trp Val Val Met Met Leu Val Ile Cys Tyr Val Leu Thr Gln Val
              915   920   925

Phe Arg Val Ser Ser Ile Thr Trp Leu Ser Glu Trp Thr Asp Ser Gly
    930   935   940

Thr Pro Lys Thr His Gly Pro Leu Phe Tyr Asn Ile Val Tyr Ala Leu
945   950   955   960

Leu Ser Phe Gly Gln Val Ser Val Thr Leu Ile Asn Ser Tyr Trp Leu
              965   970   975

Ile Met Ser Ser Leu Tyr Ala Ala Lys Lys Met His Asp Ala Met Leu
              980   985   990

Gly Ser Ile Leu Arg Ala Pro Met Val Phe Phe Gln Thr Asn Pro Leu
    995   1000   1005

Gly Arg Ile Ile Asn Arg Phe Ala Lys Asp Met Gly Asp Ile Asp Arg
    1010   1015   1020

Thr Val Ala Val Phe Val Asn Met Phe Met Gly Ser Ile Ala Gln Leu
1025   1030   1035   1040

Leu Ser Thr Val Ile Leu Ile Gly Ile Val Ser Thr Leu Ser Leu Trp
                1045   1050   1055

Ala Ile Met Pro Leu Leu Val Val Phe Tyr Gly Ala Tyr Leu Tyr Tyr
            1060   1065   1070

Gln Asn Thr Ser Arg Glu Ile Lys Arg Met Asp Ser Thr Thr Arg Ser
            1075   1080   1085

Pro Val Tyr Ala Gln Phe Gly Glu Ala Leu Asn Gly Leu Ser Ser Ile
    1090   1095   1100

Arg Ala Tyr Lys Ala Tyr Asp Arg Met Ala Glu Ile Asn Gly Arg Ser
1105   1110   1115   1120

Met Asp Asn Asn Ile Arg Phe Thr Leu Val Asn Met Ala Ala Asn Arg
                1125   1130   1135

Trp Leu Gly Ile Arg Leu Glu Val Leu Gly Gly Leu Met Val Trp Trp
            1140   1145   1150

Thr Ala Ser Leu Ala Val Met Gln Asn Gly Lys Ala Ala Asn Gln Gln
            1155   1160   1165

Ala Tyr Ala Ser Thr Met Gly Leu Leu Leu Ser Tyr Ala Leu Ser Ile
    1170   1175   1180

Thr Ser Ser Leu Thr Ala Val Leu Arg Leu Ala Ser Leu Ala Glu Asn
1185   1190   1195   1200

Ser Leu Asn Ser Val Glu Arg Val Gly Asn Tyr Ile Glu Ile Pro Ser
                1205   1210   1215

Glu Ala Pro Leu Val Ile Glu Asn Asn Arg Pro Pro Pro Gly Trp Pro
            1220   1225   1230

Ser Ser Gly Ser Ile Lys Phe Glu Asp Val Val Leu Arg Tyr Arg Pro
            1235   1240   1245

Glu Leu Pro Pro Val Leu His Gly Val Ser Phe Leu Ile Ser Pro Met
    1250   1255   1260

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Asp Lys Val Gly Ile Val Gly Arg Thr Gly Ala Gly Lys Ser Ser Leu
1265                 1270             1275             1280

Leu Asn Ala Leu Phe Arg Ile Val Glu Leu Glu Lys Gly Arg Ile Leu
                 1285             1290             1295

Ile Asp Glu Cys Asp Ile Gly Arg Phe Gly Leu Met Asp Leu Arg Lys
                 1300             1305             1310

Val Val Gly Ile Ile Pro Gln Ala Pro Val Leu Phe Ser Gly Thr Val
                 1315             1320             1325

Arg Phe Asn Leu Asp Pro Phe Ser Glu His Asn Asp Ala Asp Leu Trp
         1330             1335             1340

Glu Ser Leu Glu Arg Ala His Leu Lys Asp Thr Ile Arg Arg Asn Pro
1345             1350             1355             1360

Leu Gly Leu Asp Ala Glu Val Thr Glu Ala Gly Glu Asn Phe Ser Val
                 1365             1370             1375

Gly Gln Arg Gln Leu Leu Ser Leu Ala Arg Ala Leu Leu Arg Arg Ser
             1380             1385             1390

Lys Ile Leu Val Leu Asp Glu Ala Thr Ala Ala Val Asp Val Arg Thr
         1395             1400             1405

Asp Val Leu Ile Gln Lys Thr Ile Arg Glu Glu Phe Lys Ser Cys Thr
     1410             1415             1420

Met Leu Ile Ile Ala His Arg Leu Asn Thr Ile Ile Asp Cys Asp Lys
1425             1430             1435             1440

Val Leu Val Leu Asp Ser Gly Lys Val Gln Glu Phe Ser Ser Pro Glu
                 1445             1450             1455

Asn Leu Leu Ser Asn Gly Glu Ser Ser Phe Ser Lys Met Val Gln Ser
             1460             1465             1470

Thr Gly Thr Ala Asn Ala Glu Tyr Leu Arg Ser Ile Thr Leu Glu Asn
         1475             1480             1485

Lys Arg Thr Arg Glu Ala Asn Gly Asp Asp Ser Gln Pro Leu Glu Gly
     1490             1495             1500

Gln Arg Lys Trp Gln Ala Ser Ser Arg Trp Ala Ala Ala Ala Gln Phe
1505             1510             1515             1520

Ala Leu Ala Val Ser Leu Thr Ser Ser His Asn Asp Leu Gln Ser Leu
                 1525             1530             1535
```

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Glu Ile Glu Asp Asp Asn Ser. Ile Leu Lys Lys Thr Lys Asp Ala Val
            1540   1545   1550

Val Thr Leu Arg Ser Val Leu Glu Gly Lys His Asp Lys Glu Ile Glu
        1555   1560   1565

Asp Ser Leu Asn Gln Ser Asp Ile Ser Arg Glu Arg Trp Trp Pro Ser
    1570   1575   1580

Leu Tyr Lys Met Val Glu Gly Leu Ala Val Met Ser Arg Leu Ala Arg
1585   1590   1595   1600

Asn Arg Met Gln His Pro Asp Tyr Asn Leu Glu Gly Lys Ser Phe Asp
            1605   1610   1615

Trp Asp Asn Val Glu Met
            1620

(2) INFORMATION FOR SEQ ID NO:7:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 1251 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: double
            (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: DNA (genomic)

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

TTCACTTTTG TCCTTTTTTT CTTAACATCT ACTTTTGTCA TCAGCAAATT ATCTGTAAAT   60

AAGATAGGGT TTATGCTTAT TGCTACAATG AACCTAATCC TATGATGTGT ATTGCAATTT   120

GCAACCATGC GAGTTTAATT ATTTGTTTAC TGCTATAGTG ATCATTTTAT GATGTGTTTT   180

TATTAATTAC AAAACAGAGC ATCAAAAATC AAAAGAACAT ATCGCATAAT CGAACTATGC   240

TAATACCTCT CCTCAATCTT TGTTGTTGTT ATATTCAAGT AGCTTATTCT TTTGTTTTAT   300

TTTACGATTA GATTTCTCTA GAATTTAATT TATATTATTT AATCATACTT GATCAAGGTT   360

TGTAGCTTAA TCAATATCGT TATCGTGTCA TCCTGCAGAT TCAAATGATC AAGTCTAATA   420

ATCTACTTAT ATGTATTATA TATATTAGAT ACCACCAACG AAACAAAATC ATATTTCTAT   480

AACATTTGTT TGGTTAAATA TATTTAAAGA TTTGTAACAG TTGTTCGGGT TCAAAACTAT   540

```
CACTTTGTAG TTGTAGGATG AGGAAAAGTC GTGATATGAT CATCTACTAA AATCATGTGT        600

TTTTTAAAGA ACATGATTTT CATTGGATAG TTTAATAAAT GTTAAAAAAA TACTAAGTGT        660

CAAAGAAGAG ATTTGAACCA TATGTAGAAT ACTTGATTCG AATTTTTCCT GACGAATAAT        720

CTAATATCCT TTTCTCAAAA GAAAAAAATG TTTGTTAACT TGGACACGAT ATTATTATCC        780

AACTTCCTTT CTAGATATTC ATTTTTAAAT TACCTATATA TTTTTATTTT CTCAAAATAT        840

ACTAAAAATT GGATAGAGCT ATTAAATAAA AAGATAGAA TTTAGAGAGA AATAGCAACA         900

TAATGAATTA TAATATAAAT ATTTTGTAAA GAAATAACAA ACTTTATAGT TAGTTTGCCT        960

AATATAGAAA AAAGATACAG TTATTTACCC ATTTGTTTGT GTGTAAAAAA AGGAGTAAAA       1020

TAAACAGAGA AAAGAGCTTC TTGTTTTTAC TTGTGAACGT TATTGACTTT TCGGCCTCTC       1080

TCTCTTCTCT ATACAAATAT ATGGATCTTC ATTCTTCGT ATAGTGTAAG CAGTGACGCA       1140

TCCATTTATC ATCATCTCCT TATAAATCTC GAATCTGCCA CAGAGAGAGC GTGTGACAAA       1200

ATGAGTTCAT AAGATTCCGT TATCGTCTTC CTGATTCCTC CAAATCTCCG G              1251
```

(2) INFORMATION FOR SEQ ID NO:8:

  (i) SEQUENCE CHARACTERISTICS:
    (A) LENGTH: 1368 base pairs
    (B) TYPE: nucleic acid
    (C) STRANDEDNESS: double
    (D) TOPOLOGY: linear

  (ii) MOLECULE TYPE: DNA (genomic)

  (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

```
AAACAATTGG TGTATTTTGA ATTTTTCATG CAACGCACGT GAACAGCTTA ATTGCTTGAT         60

TGGAAACAAA CCTTTTTAGA ATTCATTAAT CAGTTTTAGG TGTTTTGGAA AATTAACGAA        120

CTATAGTGGA GATTAATTAA TTTTATATTA GTCTTTTTTA GTACACAAAT CGAAGTTTCC        180

TAGATTTTTT CAAAGTTGAA AATAATATTG ATAATATTTA TCAACAATGA ATCTACAAAA        240

ACATAATTTT TTTGCCAAAC AAATAACACC GAAACAAGAT TCATTCACTA TTTTTGGTTT        300

AAAAAAAAAA ATCAAAATTA CACTATTATG AAGCCAATTT TTGTATGCAA AAAACCTGTA        360
```

TGTATCAATT TGTTTGTATT AAAAAGTAAG CATTTATGTC TTTTTTTTAT AAATAATAGA   420

AACACTTACT AGATGAATAG ATTTTTTGGT TTTAGAACAG AATACTATAA TTGTATTTAT   480

ATAGCTTTTT TATATTATTC GATATAGAAA AGTGTTATAA TAGGAAAAAT GTACCATATA   540

CTGTCAATAA CATATTTGAT TCTAAATATA AATAGAATTG TTTTAAAGAA ATATGATCGT   600

TTATAATTAA ATGGTTTTTA ATGTCTTTTC TTGGGGCAAA AAACAAAGCT TGTCTTTCGT   660

CCATATATTT GCATCGTAAG GGGTGACGTA TCACTCTCTC TTTCTCTCAA ATATTATTCT   720

TCAATCTCTT TTTGGGGAAT CTTCGAGCAA ATTAGTGAGA GAACCCACCC ACTTTCTTTC   780

TCATATGAGT ACATAAGATC CCTTTTGAGT TTTCGTGTTT TGCCAAAATC TCCAGGTAAA   840

GCTTCTCCCT TTTTCTCTGT TTTCTCTGTT TTGTTATTCT CCCTTTTCTC CATTGTAGCT   900

TTTTCCTGTA AAGTGGGATT GATAGTTTTG TTTCATGGAT TTCAAATTTG TGTTATTTGA   960

CTCGATACCA TCTTAAATGC AGAGTCTTTT CGTGATAATA AAATTATGGA TTCGTTTCAA   1020

AGTTTTTTTT TTTTCGTATG GAAACACTT GAGCTCTCTC AATCTTGTAG TCTTGACTCT   1080

TGATGATTCT TCTATGTTCT CGTTGTGATT GCTTGTCACT GTTCTATCTT TATATATGAT   1140

TAAATGCAAT TTTGCCCCTT TTTACGCGCG AATGTATTTA TTATCTTTCG CACTCTGGGT   1200

CCATTTCTTG TCACTTGAGC ACATAATGAT TGATTTATGA CTTTTTAAAG TTATGAAAAT   1260

TTATTATTTT TGTTGCTATG GTTTTTTGGA ATTAGAAGCT CATTTCAAAG TTGTTGATTT   1320

TCTTTGCAGG GTAGGGAATT GGTGTGGTAG CTTGTGATGC ACTGTGTT   1368

(2) INFORMATION FOR SEQ ID NO:9:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 6 amino acids
            (B) TYPE: amino acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: protein

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

        Ala Cys Asp Glu Phe Gly

```
       1                5
```

(2) INFORMATION FOR SEQ ID NO:10:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 6 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: protein

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

```
Ala Cys Asp His Ile Lys
1                5
```

(2) INFORMATION FOR SEQ ID NO:11:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 12 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: double
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

```
GTGACAATAT GC                                              12
```

(2) INFORMATION FOR SEQ ID NO:12:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 54 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GTTTCACAGT TTAAAGCGTA GTCTGGGACG TCGTATGGGT AATTTTCATT GACC   54

(2) INFORMATION FOR SEQ ID NO:13:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 29 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

AAACTGCAGA TGGCTGGTAA TCTTGTTTC   29

(2) INFORMATION FOR SEQ ID NO:14:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 50 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

GCCTCTAGAT CAAGCGTAGT CTGGGACGTC GTATGGGTAA TTTTCATTGA   50

(2) INFORMATION FOR SEQ ID NO:15:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 20 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

AGATTAAGCC ATGCATGTCT   20

(2) INFORMATION FOR SEQ ID NO:16:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 23 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

TGCTGGTACC AGACTTGCCC TCC   23

(2) INFORMATION FOR SEQ ID NO:17:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 21 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

GAAAGTGGAT GTGGGACGGG C   21

(2) INFORMATION FOR SEQ ID NO:18:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 17 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

TCCATATGTT TACTGGC   17

(2) INFORMATION FOR SEQ ID NO:19:

    (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 33 base pairs
       (B) TYPE: nucleic acid
       (C) STRANDEDNESS: single
       (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

AAACCGGTGC GGCCGCCATG GGGTTTGAGC CGT   33

(2) INFORMATION FOR SEQ ID NO:20:

    (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 20 base pairs
       (B) TYPE: nucleic acid
       (C) STRANDEDNESS: single
       (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

AATTAACCCT CACTAAAGGG   20

What is claimed is:

1. An isolated DNA encoding a plant GS-X pump polypeptide.

2. The isolated DNA of claim 1, wherein said DNA is selected from the group consisting of DNA comprising *AtMRP1* and *AtMRP2*, and any mutants, derivatives, homologs and fragments thereof encoding GS-X pump activity.

3. An isolated preparation of a polypeptide comprising a plant GS-X pump.

4. The isolated preparation of a polypeptide of claim 3, wherein said polypeptide is selected from the group consisting of AtMRP1, AtMRP2, and any mutants, derivatives, homologs and fragments thereof having GS-X pump activity.

5. A recombinant cell comprising the isolated DNA of claim 1.

6. The recombinant cell of claim 5, wherein said cell is selected from the group consisting of a prokaryotic cell and a eukaryotic cell.

7. A vector comprising the isolated DNA of claim 1.

8. An antibody specific for a plant GS-X pump polypeptide.

9. An isolated preparation of a nucleic acid which is in an antisense orientation to all or a portion of a plant GS-X pump gene.

10. A cell comprising the isolated preparation of a nucleic acid of claim 9.

11. A vector comprising the isolated preparation of a nucleic acid of claim 9.

12. A transgenic plant, the cells, seeds and progeny of which comprise an isolated DNA encoding a plant GS-*X* pump.

13. A transgenic plant, the cells, seeds and progeny of which comprise an isolated preparation of a nucleic acid which is in an antisense orientation to all or a portion of a plant GS-*X* pump gene.

14. A transgenic plant, the cells, seeds and progeny of which comprise an isolated DNA encoding YCF1, or any mutants, derivatives, homologs and fragments thereof having YCF1 activity.

15. An isolated DNA comprising a plant GS-X pump promoter sequence.

16. The isolated DNA of claim 15, wherein said promoter sequence is selected from the group consisting of an *AtMRP1* and an *AtMRP2* promoter sequence.

17. A cell comprising the isolated DNA of claim 15.

18. A vector comprising the isolated DNA of claim 15.

19. The isolated DNA of claim 15, further comprising a reporter gene operably fused thereto.

20. A transgenic plant, the cells, seeds and progeny of which comprise a transgene comprising an isolated DNA comprising GS-*X* pump promoter sequence.

21. A method of identifying a compound capable of affecting the expression of a plant GS-*X* gene comprising

providing a cell comprising an isolated DNA comprising a plant GS-X pump promoter sequence having a reporter sequence operably linked thereto,

adding to said cell a test compound, and

measuring the level of reporter gene activity in said cell, wherein a higher or a lower level of reporter gene activity in said cell compared with the level of reporter gene activity in a cell to which the test compound was not added, is an indication that said test compound is capable of affecting the expression of a plant GS-*X* pump gene.

22. A method of removing xenobiotic toxins from soil comprising growing in the soil a transgenic plant of comprising an isolated DNA encoding a GS-*X* pump.

23. A method of removing heavy metals from soil comprising growing in the soil a transgenic plant of comprising an isolated DNA encoding a GS-*X* pump.

24. A method of generating a transgenic pathogen resistant plant comprising introducing to the cells of said plant an isolated DNA encoding a GS-*X* pump, wherein said pump is capable of transporting glutathionated isoflavonoid alexins into the cells of said plant.

25. A method of manipulating plant pigmentation comprising modulating the expression of a GS-*X* pump protein in said plant, wherein said GS-*X* pump protein is selected from the group consisting of AtMRP1, AtMRP2 and YCF1.

26. A method of alleviating oxidative stress in a plant comprising introducing into the cells of said plant DNA encoding a GS-X pump, wherein said DNA is selected from the group consisting of DNA encoding AtMRP1, AtMRP2 and YCF1.

27. A method of manipulating the expression of a gene in a plant cell comprising

operably fusing a GS-X pump promoter sequence to the DNA sequence encoding said gene to form a chimeric DNA, and

generating a transgenic plant, the cells of which comprise said chimeric DNA, wherein upon activation of said GS-X pump promoter sequence, the expression of said gene is manipulated.

Fig. 1A

Fig. 1B

Fig. 2
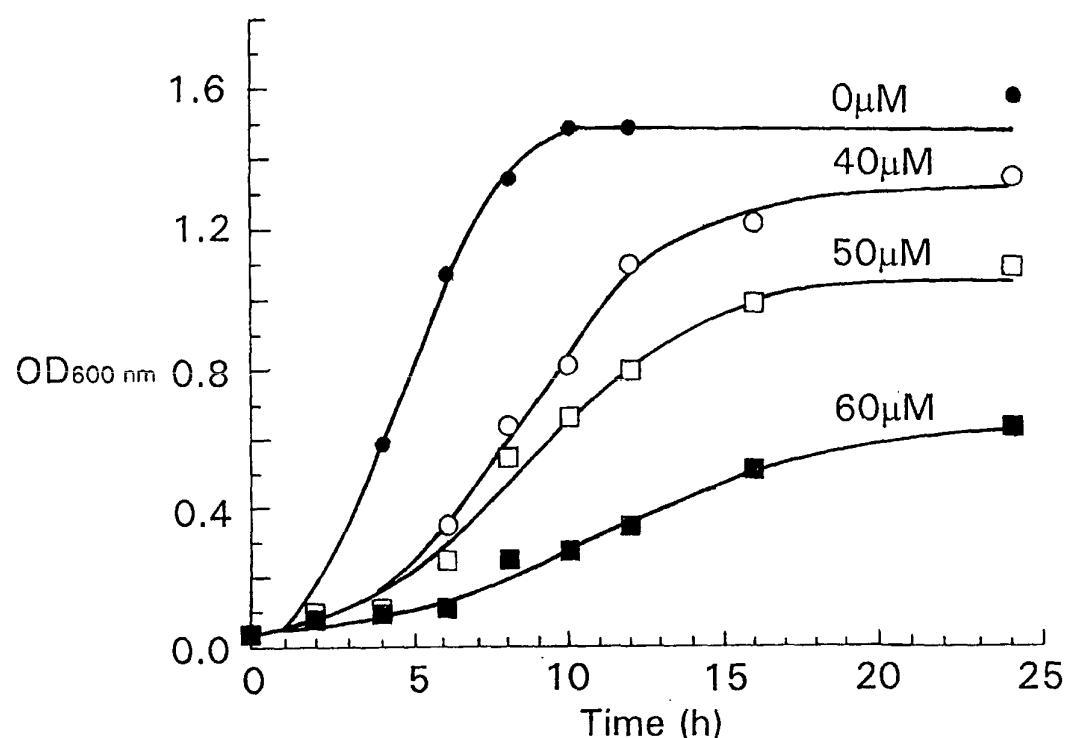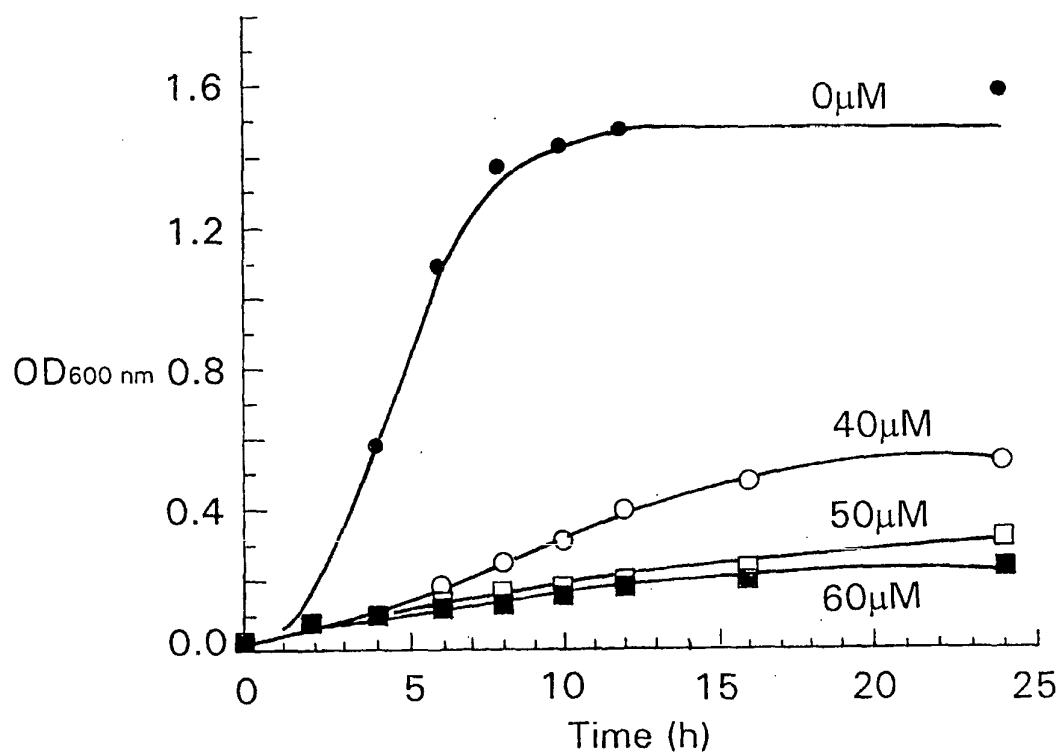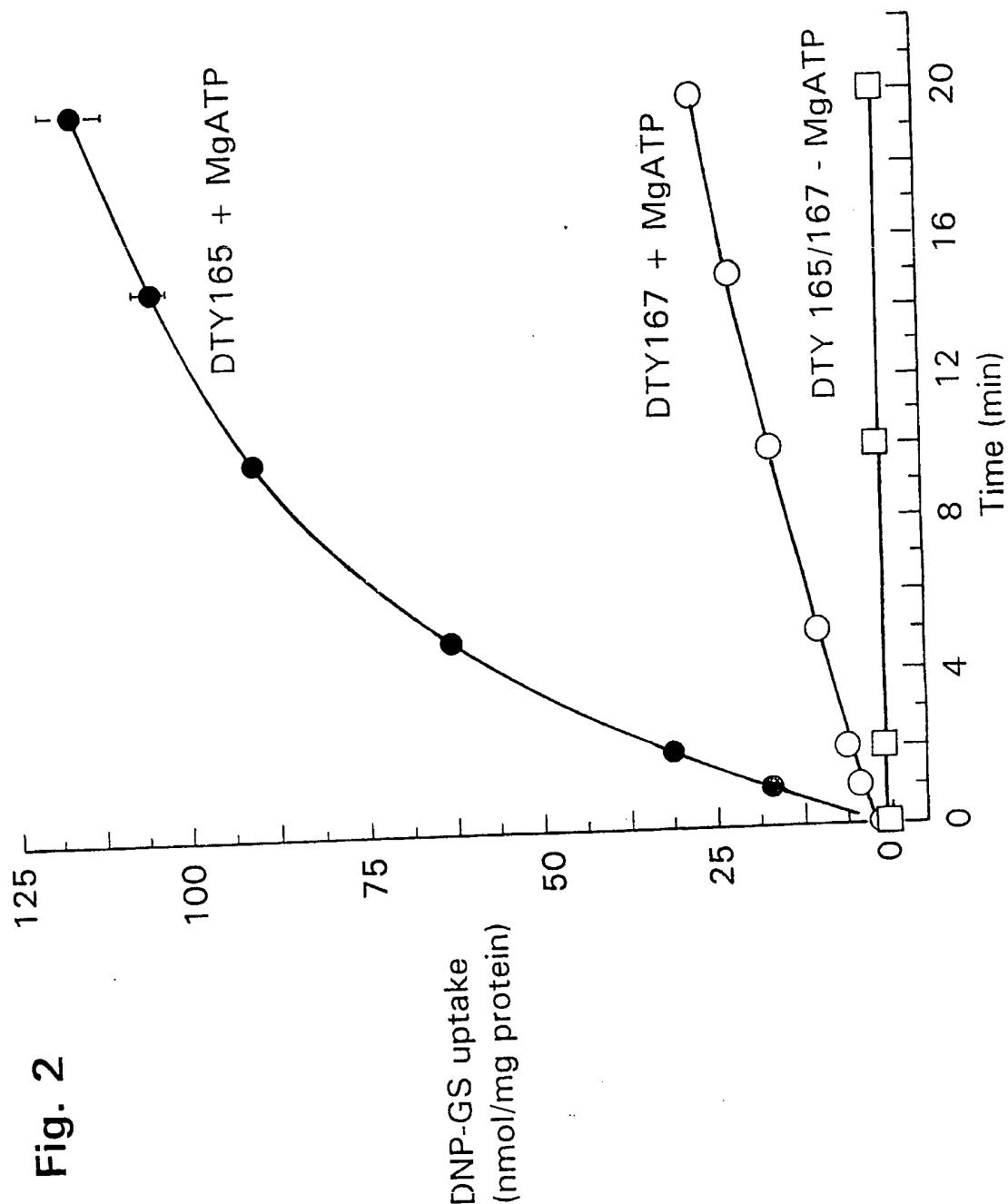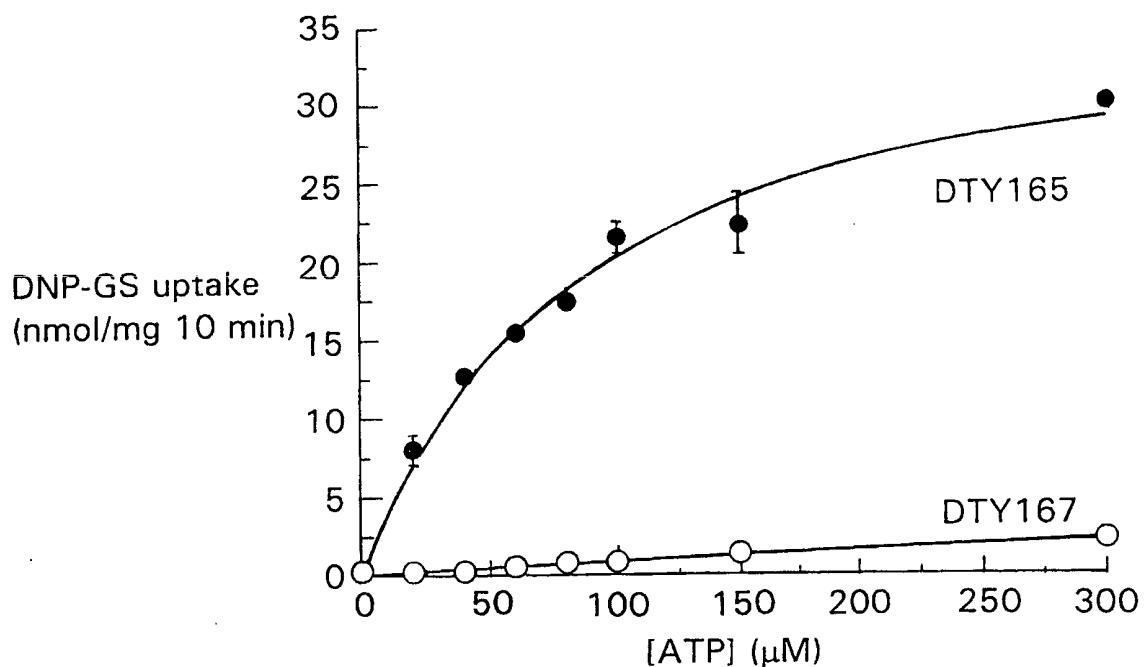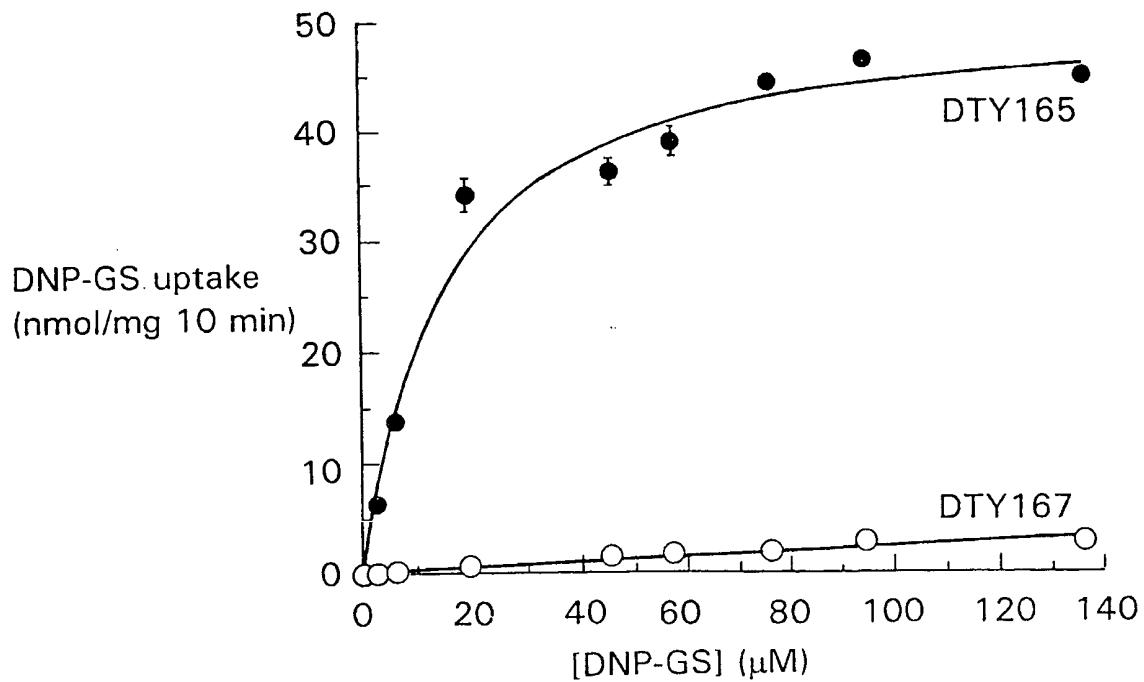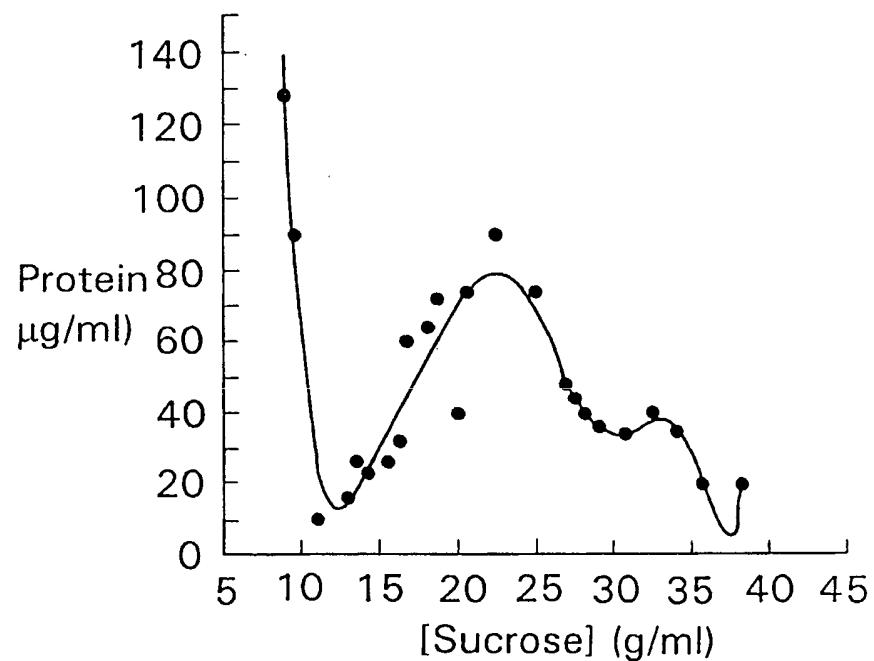
Fig. 3A

Fig. 3B

Fig. 4A

Fig. 4B
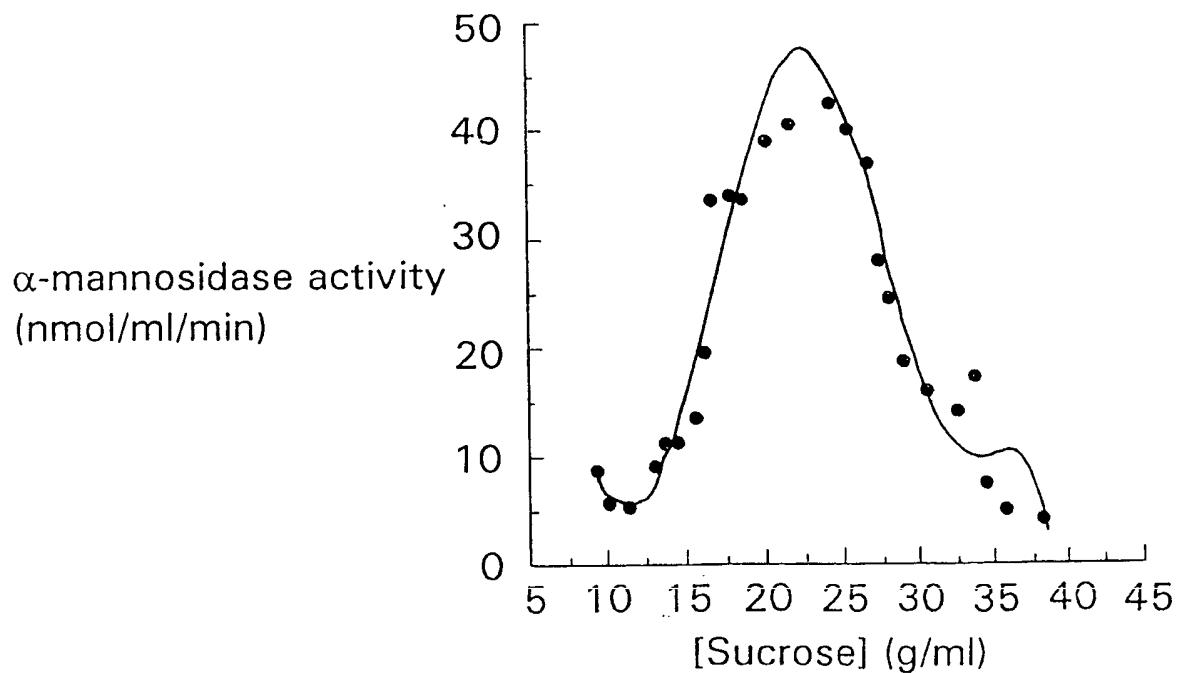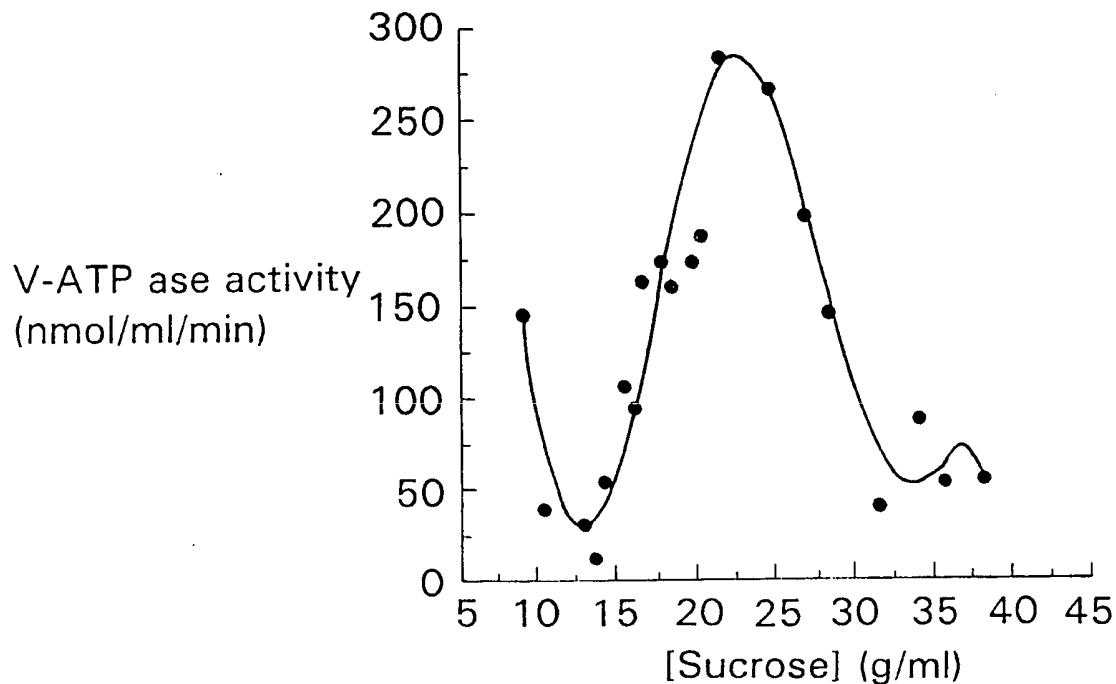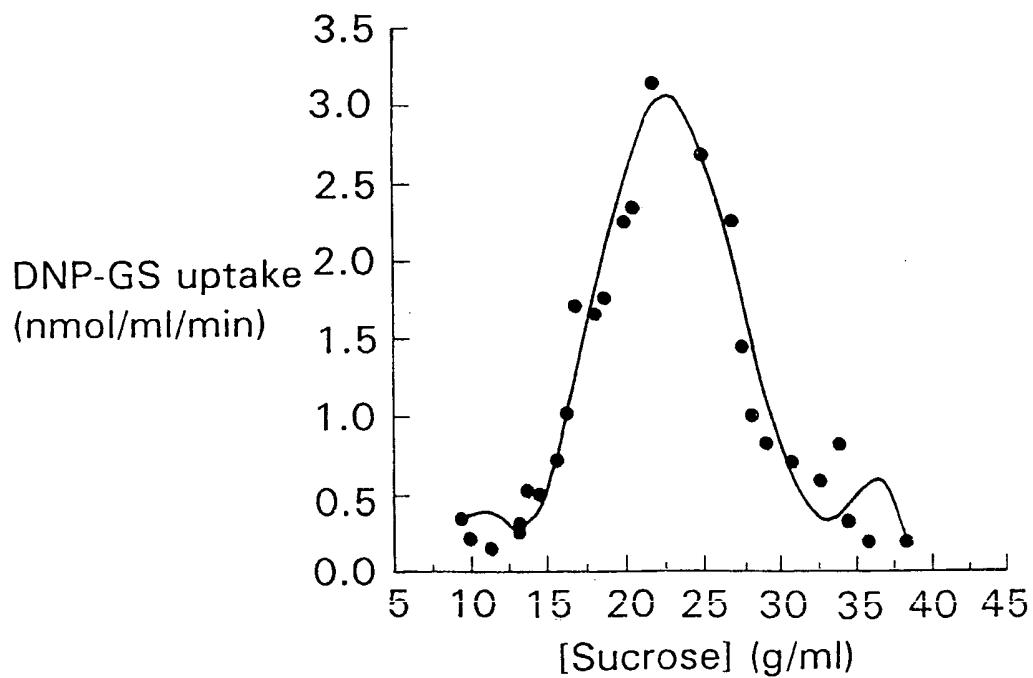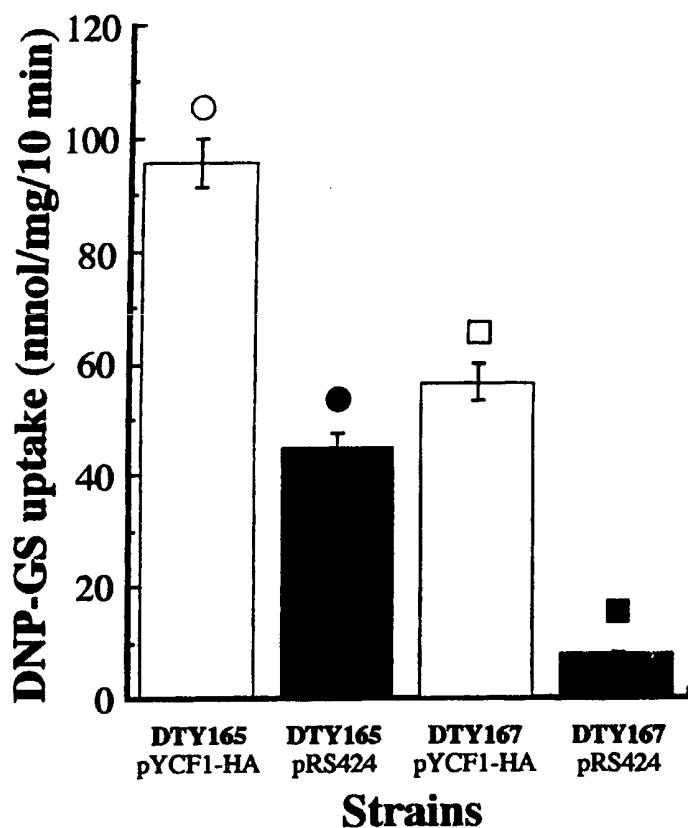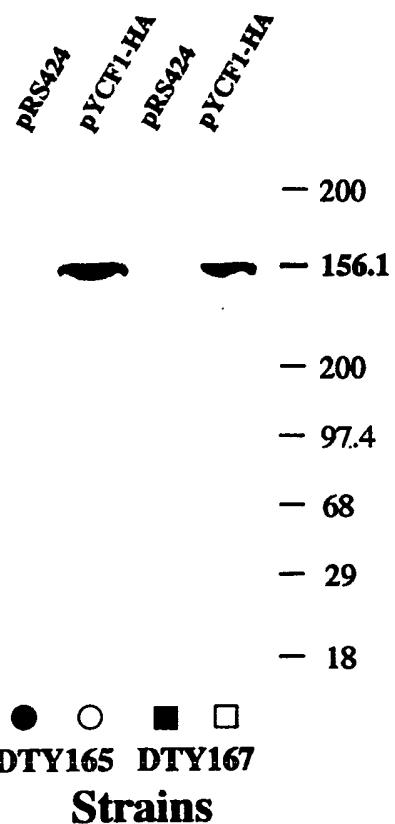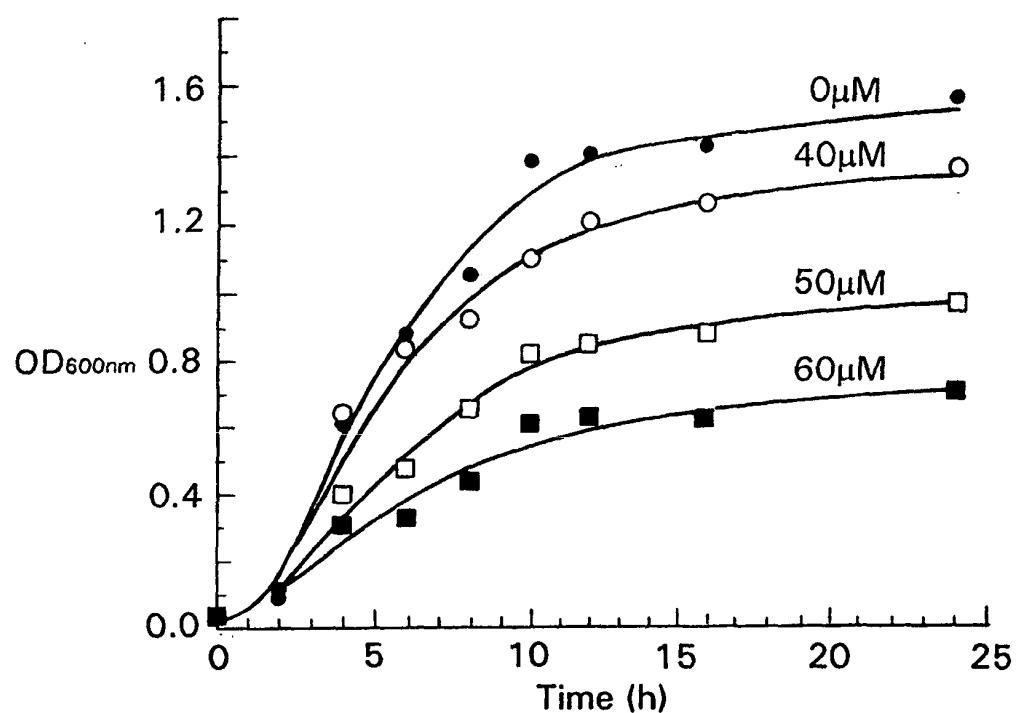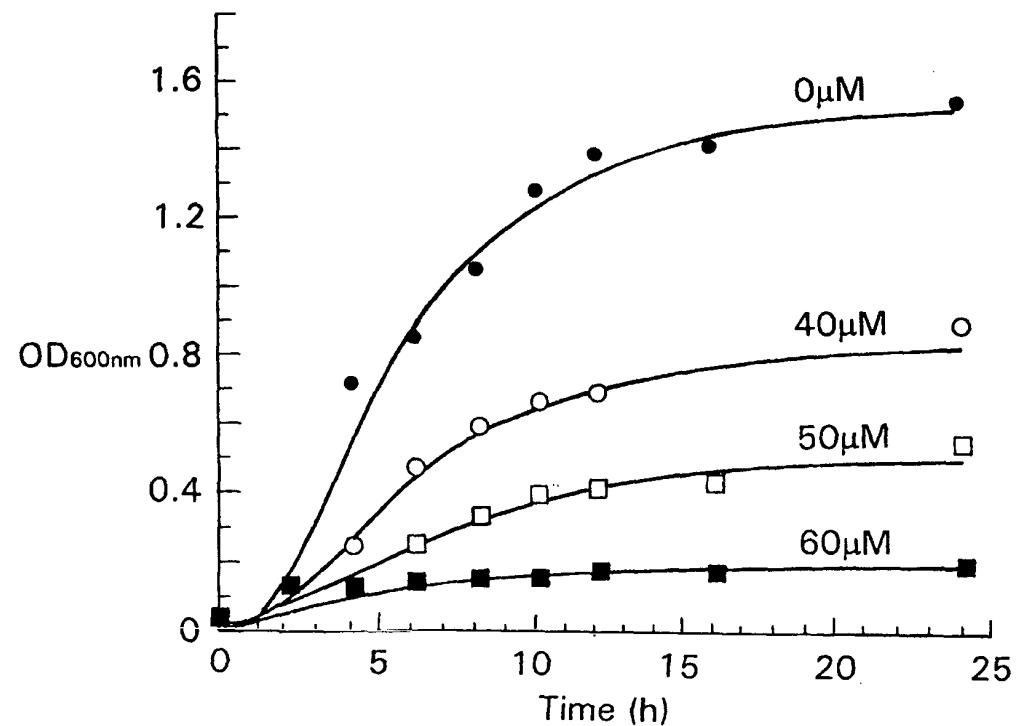
Fig. 4C

V-ATP ase activity
(nmol/ml/min)

[Sucrose] (g/ml)

Fig. 4D

DNP-GS uptake
(nmol/ml/min)

[Sucrose] (g/ml)

Fig. 5A

Fig. 5B

Fig. 6A

Fig. 6B
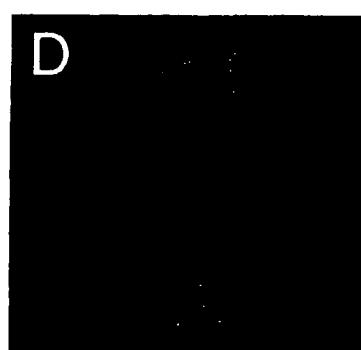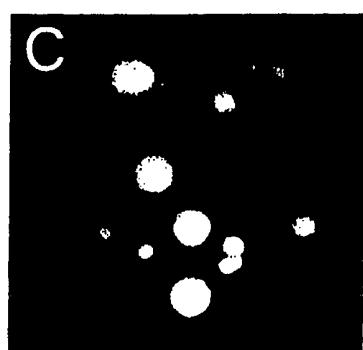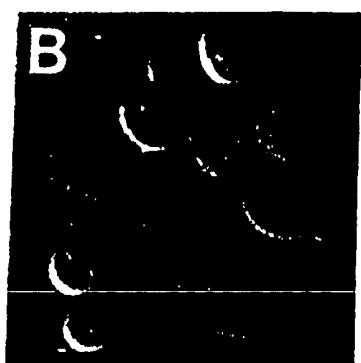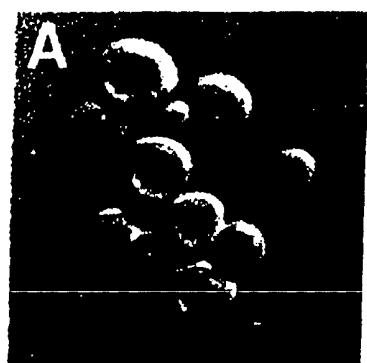
Fig. 7

Fig. 8A
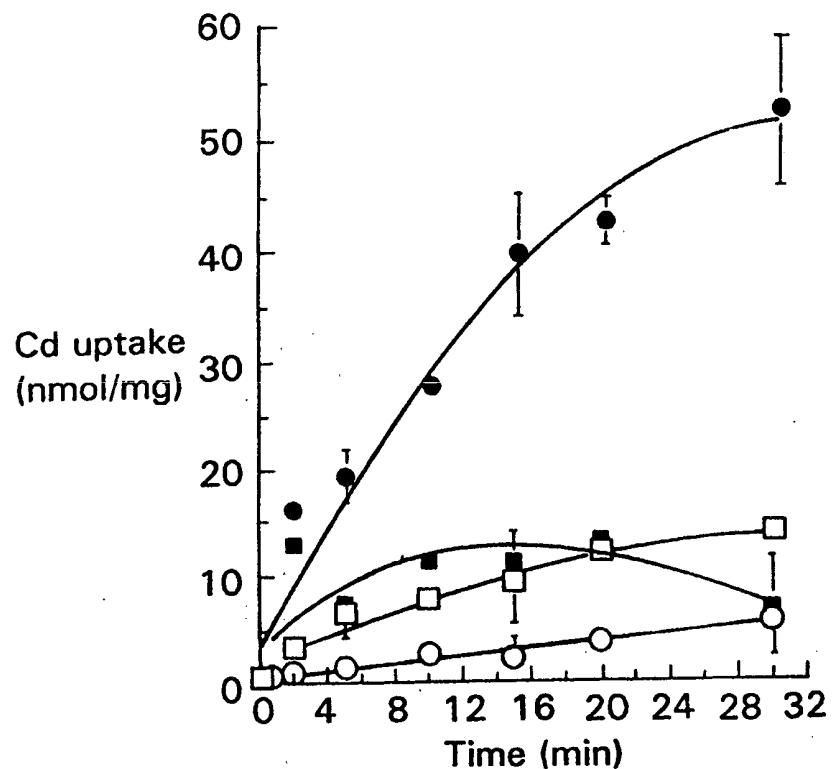
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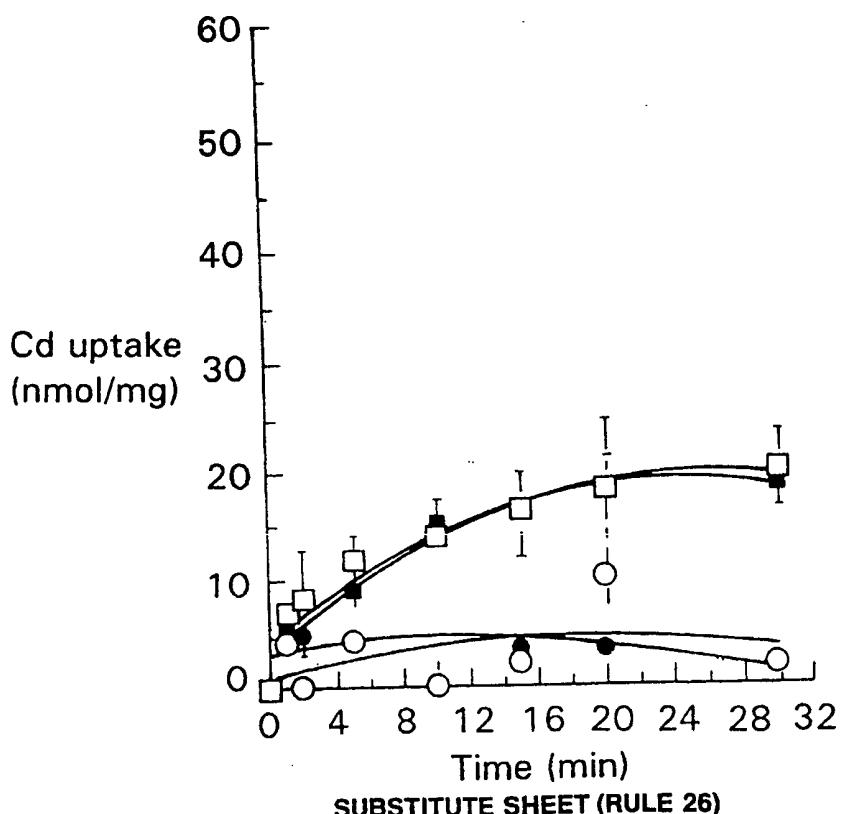
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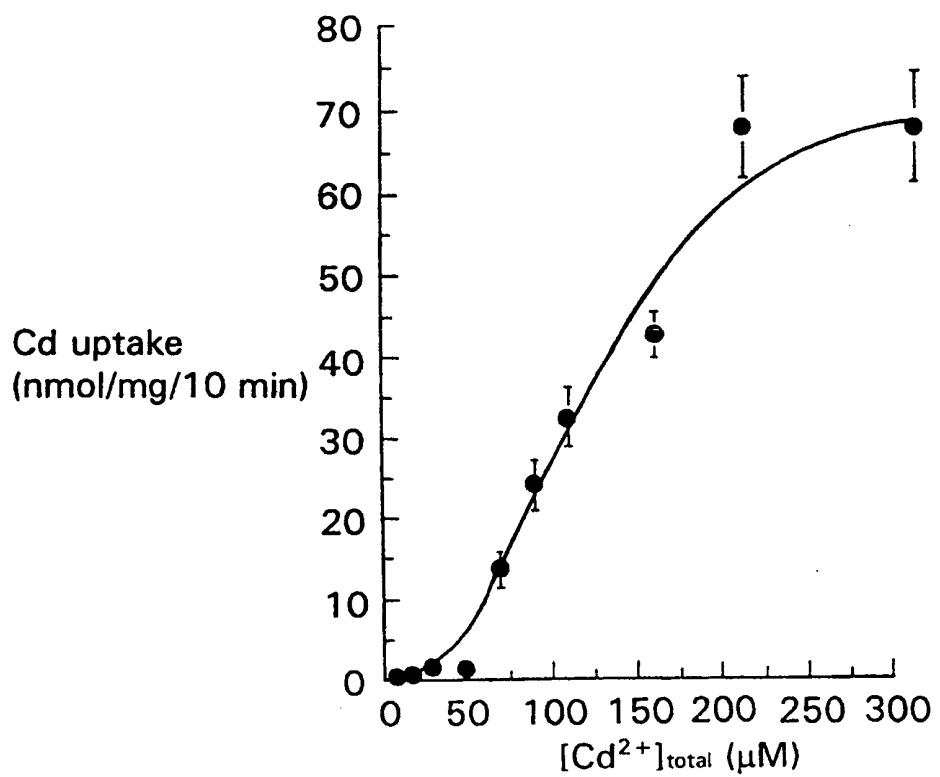
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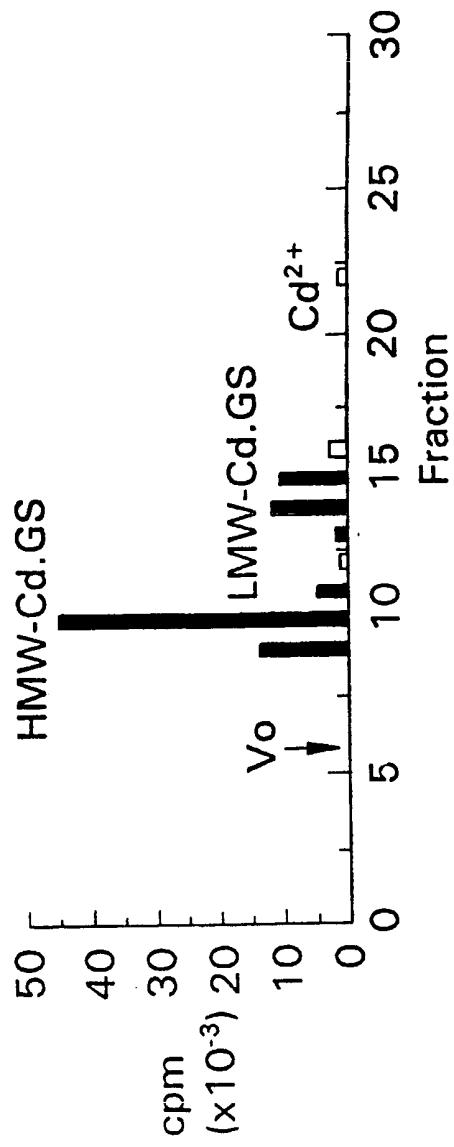
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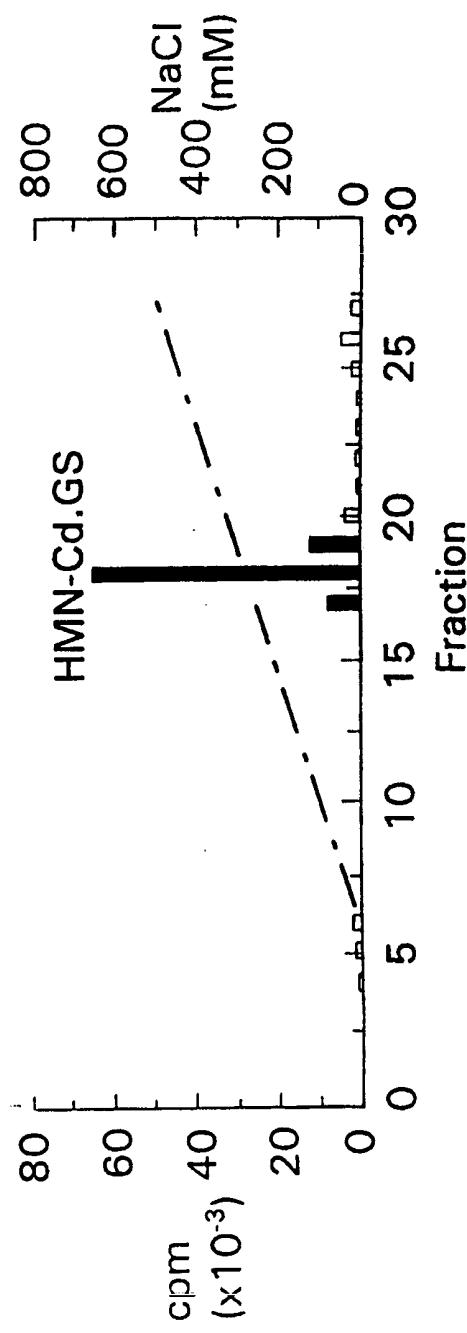
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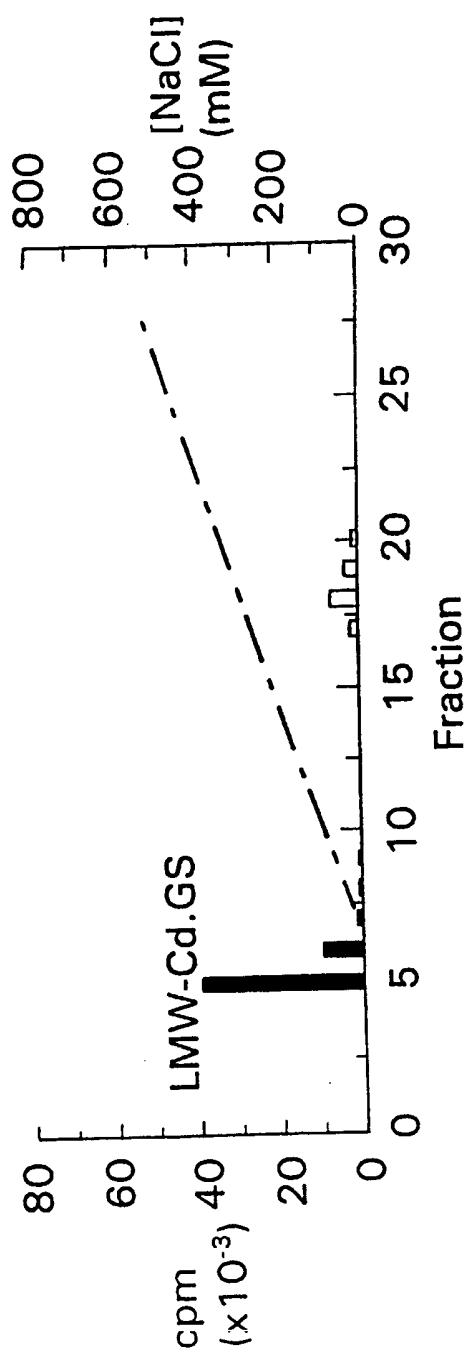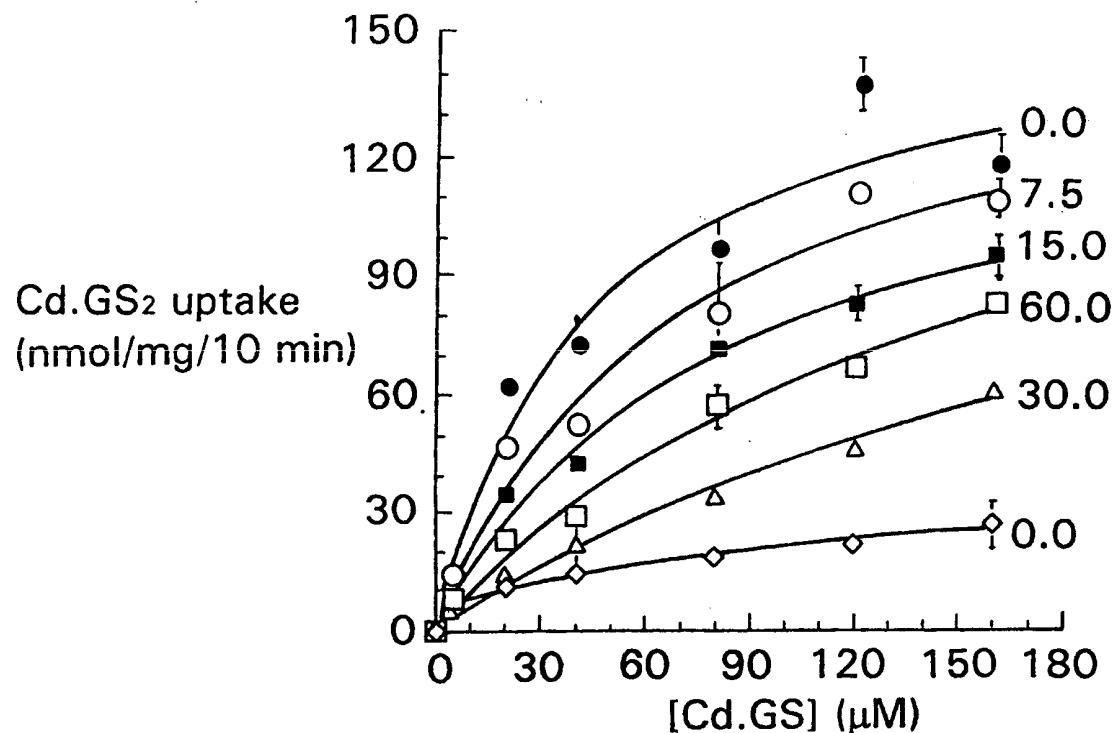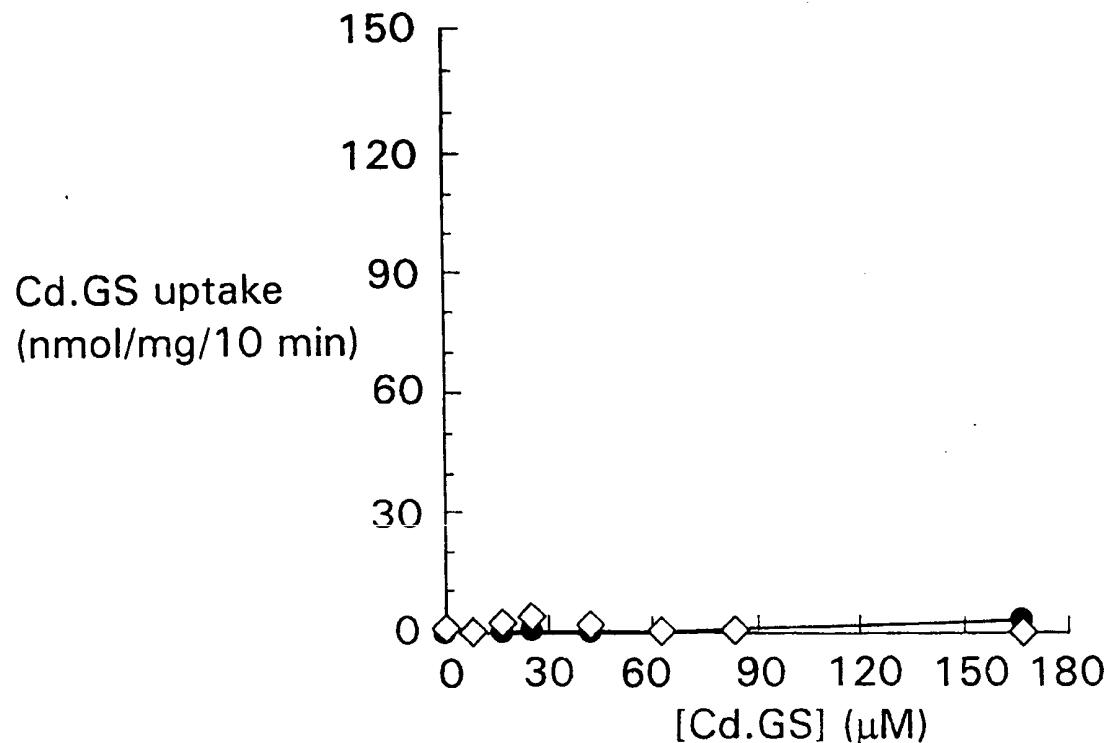
Fig. 10A

Fig. 10B

Fig. 10C
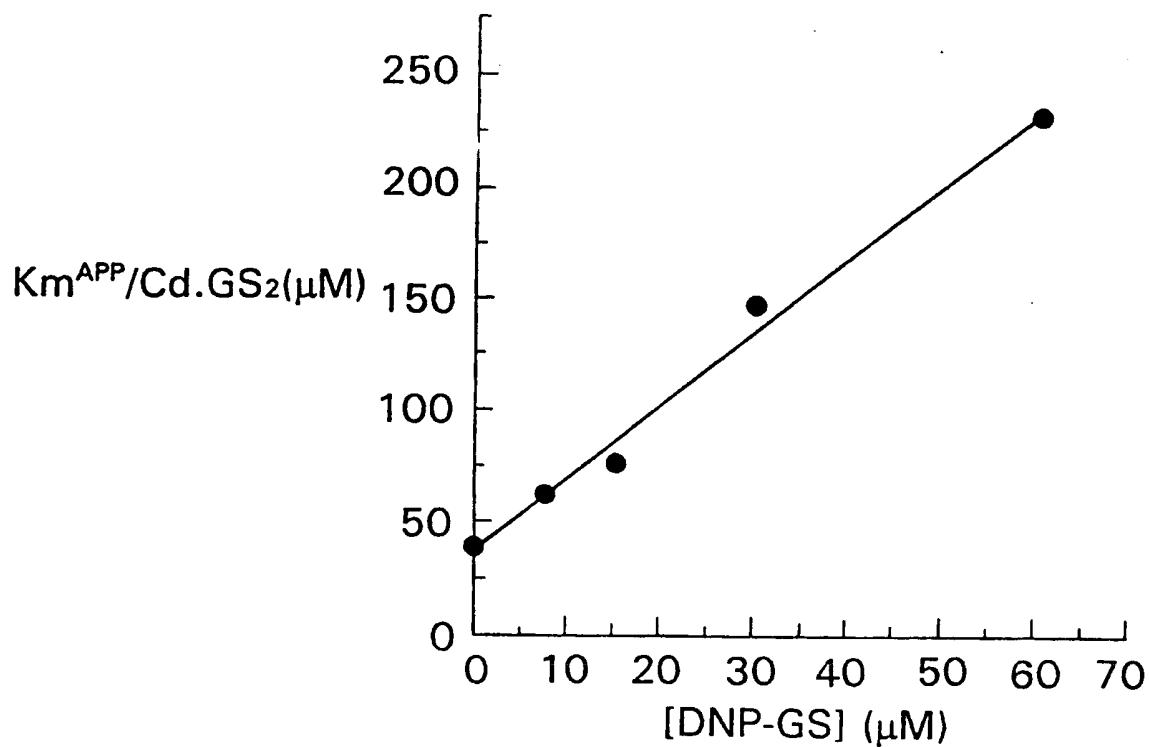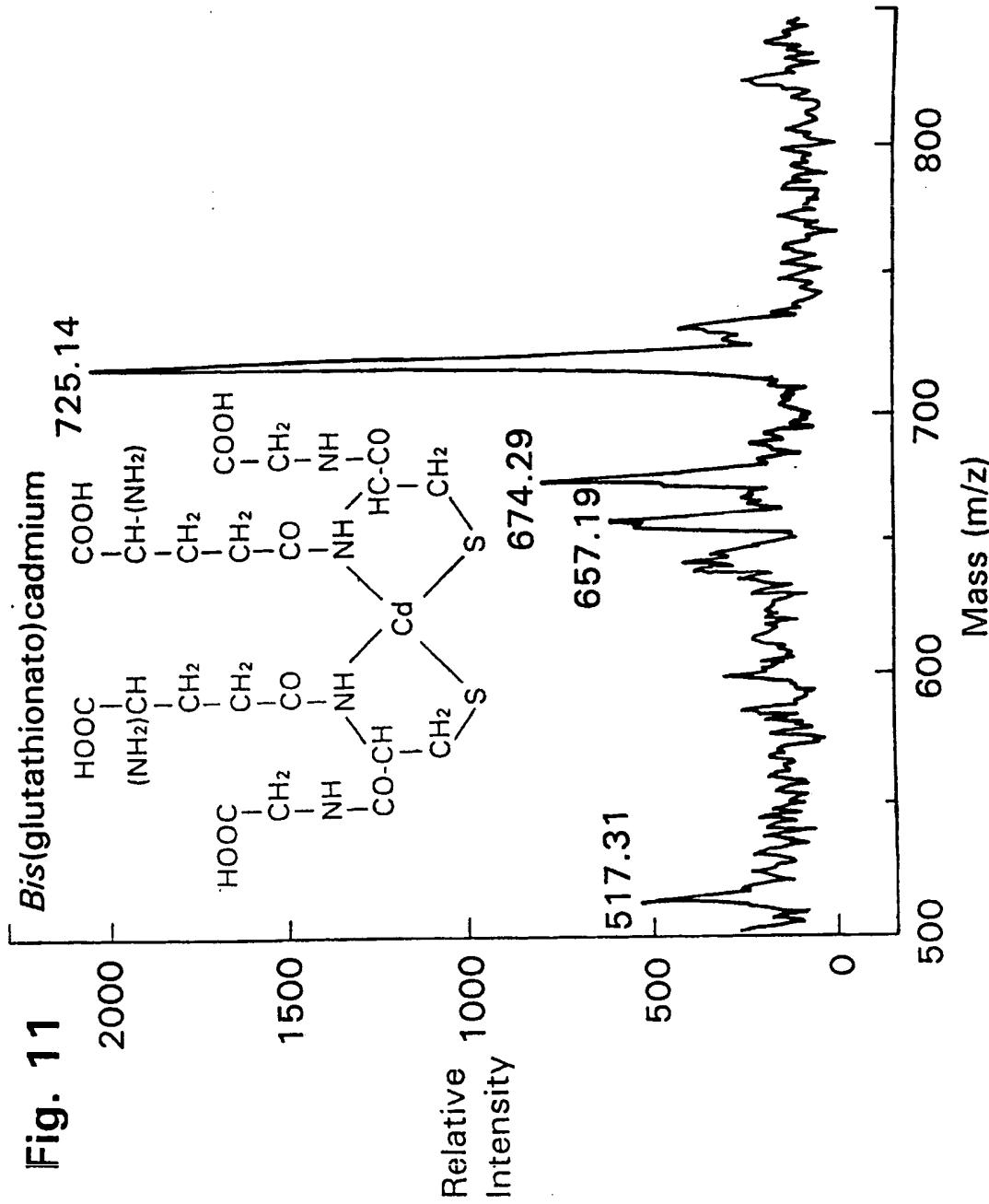
Fig. 11

Fig. 12

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```

FIGURE 13A

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FIGURE 13B

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FIGURE 14A

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GCAATGGATACAGTAAAgtaagaaattctagaaccaattttgttaacatagttattaatt
tgcaggaaacttgtactaaaccaaaatgctacagGTGTTATGCTTGGGAAAACAGTTTCC
AGTCCAAGGTCCAAACTGTCGTGATGATGAATTATCTTGGTTCCGGAAATCACAGCTCCT
GGGAGCGgtatgactacagcgtagttacttttgttttttcctctaattattgtatatttct
aactcttgcttggtcttgtcttgttttgcagTTGAATATGTTCATACTGAATAGCATTCC
TGTTCTTGTGACTATTGTTTCATTTGGTGTGTTCACATTACTTGGAGGAGACCTGACCCC
TGCAAGAGCATTTACGTCACTCTCTCTCTTTGCTGTGCTTCGTTTCCCTCTCTTCATGCT
TCCAAACATTATAACTCAGgtgatttcttaaatatgttgttgcaatgcatgtgtattaag
tagaactgttagtgcttgtagtaactgtcgtttggttatcaaatccatgacttatatttc
gaatttacatgctggagggtatccttgctggtgccagaaacagatgccgatgctgactag
ttttcacttgtagGTGGTAAATGCTAATGTATCCTTAAAACGTCTTGAGGAGGTATTGGC
GACAGAAGAAAGAATTCTCTTACCAAATCCTCCCATTGAACCTGGAGAGCCAGCCATCTC
AATAAGAAATGGATATTTCTCTTGGGATTCTAAGgtgtcgcttggctattctataccatg
ttccttctttcgcttctctcattacctttatccatagaaagtacaaaaatcgagctaacc
ctatgtatctacagGGGGATAGGCCGACGTTGTCAAATATCAACTTGGATGTACCTCTTG
GCAGCCTAGTTGCTGTGGTTGGTAGTACAGGCGAAGGAAAAACCTCTCTAATATCTGCTA
TCCTTGGTGAACTTCCTGCAACATCTGATGCAATAGTTACTCTCAGAGGATCAGTTGCTT
ATGTTCCACAAGTTTCATGGATCTTTAATGCAACAgtatgttcttctttctttgacttt
taagttgggctgacgttgcaaattttctgttgtacataatgttaaatgtattttctgtc
ttttatagtagaacaatatgtgttctcaaatgcgtcagttacttcaccaacttagtggaa
accttcttcaatatttgattctctaagctattttgaacagaagactgatatgcattttct
tataaaaatttgtagGTACGCGACAATATACTGTTTGGTTCTCCTTTCGACCGTGAAAAG
TATGAAAGGGCCATTGATGTGACTTCACTGAAGCATGACCTAGAGTTACTGCCTgtaagt
tttgaggagagcttcgtggagttgataacaaggatttgtcttgcctgttctcgtgttgct

FIGURE 14B

aagtttgtttcaacctctttctcttgcttaatagGGTGGTGATCTCACGGAGATTGGAGA
AAGAGGTGTTAATATCAGTGGAGGACAGAAGCAGAGGGTTTCCATGGCTAGGGCCGTTTA
CTCAAATTCAGATGTGTACATCTTTGATGACCCGTTAAGTGCCCTTGATGCTCATGTTGG
TCAACAGgtactaactcattgattctctttgataaggctagtctatttcatttttgaatt
tatctaacattttttgtgtctggtcattatgggaatactgtcagtctgatttctaggaata
ttgtttcagGTTTTTGAAAAATGCATAAAAAGAGAACTGGGGCAGAAAACGAGAGTTCTT
GTTACAAACCAGCTCCACTTCCTATCACAAGTGGACAGAATTGTACTTGTGCATGAAGGC
ACAGTGAAAGAGGAAGGAACATATGAAGAGCTATCCAGTAATGGGCCTTTGTTCCAGAGG
GTAATGGAAAATGCAGGGAAGGTGGAAGAATATTCAGAAGAAAATGGAGAAGCTGAGGCA
GACCAAACAGCGGAACAACCAGTTGCGAATGGGAACACAAATGGTCTTCAAATGGATGGA
AGTGACGATAAAAAATCCAAAGAAGGAAATAAAAAGGAGGGAAATCTGTCCTCATCAAG
CAAGAAGAACGTGAAACCGGAGTTGTAAGTTGGAGAGTCCTGAAGAGgtaacttgaacat
ttggcttttgcaatcttactatttgtttgcaactttccccatactcgatccaagaggtcc
attcatttgtggtgtgtttcacaacaaactagcatgttccttatgttttttaggctgaactat
accttttgcgggatatcagaatgacttttccaggctttcaatgttttcagGTACCAGGATG
CACTTGGAGGGGCATGGGTAGTGATGATGCTCCTTTTATGTTACGTCTTAACAGAAGTAT
TTCGGGTTACTAGCAGCACGTGGTTGAGTGAGTGGACTGATGCAGGAACTCCAAAGAGTC
ATGGACCCCTTTTCTACAATCTCATATATGCACTTCTCTCGTTTGGACAGgtatgagtta
tgtttgcttgatggatgagtgaagatttgatataatcttgacctcatgatataacatata
tagctgaaacctgaccagcttagaaagatcttatataattctactttgtgattttactt
tgagaatccaaaggtggaggtagaaaaggttagtaaagaattgattttttgctgagact
ctttcttcttgcttacagGTTTTGGTGACATTGACCAATTCATATTGGTTGATTATGTCC
AGTCTTTATGCAGCTAAGAAGTTACACGACAATATGCTTCATTCCATACTGAGGGCCCCG
ATGTCCTTCTTCCATACCAATCCGCTAGGACGGATAATCAATCGATTCGCAAAAGATCTG
GGTGATATTGATCGAACTGTGGCCGTCTTTGTAAACATGTTTATGGGTCAAGTCTCACAG
CTTCTTTCAACTGTAGTGTTGATTGGCATTGTAAGCACTTTGTCCTTGTGGGCCATCATG
CCCCTCCTGGTCTTGTTTTATGGAGCTTATCTTTATTATCAGtaatgtaccttctgacc
gcagcatttaaataactgagattaagtgacagaaagagaaaaggacacagatgatggatg
ttacacatacttttttagcctcatttgtcatgtctgagttcgtttggtgcttaagctatc
tacactcatctgtcaccaaaaatcatgctgtatatgttgtgtgttaaatattttttcttat
tgcagAACACAGCCCGTGAGGTTAAGCGTATGGATTCAATTTCAAGATCGCCTGTTTATG
CACAGTTTGGAGAGGCATTGAATGGCTTATCAACTATCCGTGCTTACAAAGCATATGATC
GTATGGCTGATATCAACGGAAGATCAATGGATAATAACATCAGATTCACTCTTGTCAACA
TGGGTGCCAATCGGTGGCTTGGAATCCGTTTAGAAACTCTGGGTGGTCTTATGATATGGC
TGACAGCATCGTTTGCTGTCATGCAGAATGGAAGAGCGGAGAACCAACAGGCATTTGCAT
CTACAATGGGTTTGCTTCTCAGTTATGCCTTAAATATTACTAGCTTGTTAACAGGTGTTC
TGAGACTTGCGAGTTTGGCTGAGAATAGTCTAAACGCGGTCGAGTGTTGGCAATTATATA
GAGATTCCGCCAGAGGTCCGCCTGTCATTGAGAACAACCGTCCACCTCCTGGATGGCCAT
CATCTGGATCCATAAAGTTTGAGGATGTTGTTCTCCGTTACCGCCCTCAGTTACCGCCTG
TGCTTCATGGGGTTTCTTTCTTCATTCATCCAACAGATAAGGTGGGGATTGTTGGAAGGA
CTGGTGCTGGAAAGTCAAGCCTGTTGAATGCATTGTTTAGAATTGTGGAGGTGGAAAAAG
GAAGGATCTTAATCGATGATTGTGACGTTGGAAAGTTTGGACTGATGGACCTACGTAAAG
TGCTCGGAATCATTCCACAGTCACCGGTTCTTTTCTCAGGAACTGTGAGGTTCAATCTTG
ATCCATTTGGTGAACACAATGATGCTGATCTTTGGGAATCTCTAGAGAGGGCACACTTGA
AGGATACCATCCGCAGAAATCCTCTTGGTCTTGATGCTGAGgtattcagttgctgcctat
attgatatgaagtctcattttttaagtggtaataactgattttcaatctttgttcagGTC
TCTGAGGCAGGAGAGAATTTCAGCGTGGGACAGAGGCAATTGTTGAGTCTTTCACGTGCG
CTGTTACGGAGATCTAAGATACTCGTCCTTGATGAAGCAACTGCTGCTGTAGATGTTAGA
ACCGATGCCCTCATTCAGAAGACTATCCGAGAAGAATTCAAGTCATGCACGATGCTCATT

FIGURE 14C

ATCGCTCACCGTCTCAATACCATCATTGACTGTGACAAAATTCTCGTGCTTGATTCTGGA
AGAgtatgatttttaaacactctctctctttcaatctcacactctccttgtttctcagcta
acctgttctattccaatttgttaactcagGTTCAAGAATTCAGTTCACCGGAGAACCTTC
TTTCAAATGAAGGAAGCTCTTTCTCCAAGATGGTTCAAAGCACTGGAGCTGCAAATGCTG
AGTACTTGCGTAGTTTAGTACTCGACAACAAGCGTGCCAAAGATGACTCACACCACTTAC
AAGGCCAAAGGAAATGGCTGGCTTCTTCTCGCTGGGCTGCAGCCGCTCAGTTTGCTCTGG
CTGCGAGTCTTACTTCGTCGCACAACGATCTTCAAAGCCTTGAAATTGAAGATGACAGCA
GCATTTTGAAGAGAACAAACGATGCAGTTGTGACTCTGCGCAGTGTTCTCGAGGGGAAAC
ACGACAAAGAGATTGCAGAGTCGCTTGAGGAACATAATATCTCTAGAGAGGGATGGTTGT
CATCACTCTATAGAATGGTAGAAGgtaaaccaaatatgcatctctacaaatgcttatgca
aaatcttaatcaccacactgaaacattaaagtcaaatcgtgctcttatattgcaagcctg
ctttccgctgtctacgtttcagGGCTTGCAGTGATGAGCAGATTGGCAAGGAACCGAATG
CAACAACCGGATTACAATTTCGAAGGAAATACATTTGACTGGGACAACGTCGAGATGTAG
ATAAGTTCATGTTAAACTAGGAATCATTGTCTCTTCCGTAAGAAACATATATTTATCTTA
ACCAAAATTATTAGTTTGGTTTCCATTTCATAAACTTAATTTTCACCTGCAAAGAAAATC
AAACCCTGTTGTGTTCTTCGTGATAAGTAGAGAAATTACTTGAGTATCCTTCTAACTCat
aaatgggatctcatgattcatgaacaagcagcaacacaataatacccttttcagattttg
gagctggacaaagttgttaagttgagtttctcttacagtcattcatatacaaaaacctct
tcgactgaagcaccaagaaagaaacaaacatcaaaagggaatgaggtcttttcttagggc
tgagatcatcggaatgtgggagtgcggaacacgacc

FIGURE 14D

MGFEFIEWYCKPVPNGVWTKTVANAFGAYTPCATDSFVLGISQLVLLVLCLYRIWLALKD
HKVERFCLRSRLYNYFLALLAAYATAEPLFRLIMGISVLDFDGPGLPPFEAFGLGVKAFA
WGAVMVMILMETKIYIRELRWYVRFAVIYALVGDMVLLNLVLSVKEYYSSYVLYLYTSEV
GAQVLFGILLFMHLPNLDTYPGYMPVRSETVDDYEYEEISDGQQICPEKHPNIFDKIFFS
WMNPLMTLGSKRPLTEKDVWYLDTWDQTETLFTSFQHSWDKELQKPQPWLLRALNNSLGG
RFWWGGFWKIGNDCSQFVGPLLLNQLLKSMQEDAPAWMGYIYAFSIFGGVVFGVLCEAQY
FQNVMRVGYRLRSALIAAVFRKSLRLTNEGRRKFQTGKITNLMTTDAESLQQICQSLHTM
WSAPFRIIIALILLYQQLGVASLIGALLLVLMFPLQTVIISKMQKLTKEGLQRTDKRIGL
MNEVLAAMDTVKCYAWENSFQSKVQTVRDDELSWFRKSQLLGALNMFILNSIPVLVTIVS
FGVFTLLGGDLTPARAFTSLSLFAVLRFPLFMLPNIITQVVNANVSLKRLEEVLATEERI
LLPNPPIEPGEPAISIRNGYFSWDSKGDRPTLSNINLDVPLGSLVAVVGSTGEGKTSLIS
AILGELPATSDAIVTLRGSVAYVPQVSWIFNATVRDNILFGSPFDREKYERAIDVTSLKH
DLELLPGGDLTEIGERGVNISGGQKQRVSMARAVYSNSDVYIFDDPLSALDAHVGQQVFE
KCIKRELGQKTRVLVTNQLHFLSQVDRIVLVHEGTVKEEGTYEELSSNGPLFQRLMENAG
KVEEYSEENGEAEADQTAEQPVANGNTNGLQMDGSDDKKSKEGNKKGGKSVLIKQEERET
GVVSWRVLKRYQDALGGAWVVMMLLLCYVLTEVFRVTSSTWLSEWTDAGTPKSHGPLFYN
LIYALLSFGQVLVTLTNSYWLIMSSLYAAKKLHDNMLHSILRAPMSFFHTNPLGRIINRF
AKDLGDIDRTVAVFVNMFMGQVSQLLSTVVLIGIVSTLSLWAIMPLLVLFYGAYLYYQNT
AREVKRMDSISRSPVYAQFGEALNGLSTIRAYKAYDRMADINGRSMDNNIRFTLVNMGAN
RWLGIRLETLGGLMIWLTASFAVMQNGRAENQQAFASTMGLLLSYALNITSLLTGVLRLA
SLAENSLNAVERVGNYIEIPPEAPPVIENNRPPPGWPSSGSIKFEDVVLRYRPQLPPVLH
GVSFFIHPTDKVGIVGRTGAGKSSLLNALFRIVEVEEGRILIDDCDVGKFGLMDLRKVLG
IIPQSPVLFSGTVRFNLDPFGEHNDADLWESLERAHLKDTIRRNPLGLDAEVSEAGENFS
VGQRQLLSLSRALLRRSKILVLDEATAAVDVRTDALIQKTIREEFKSCTMLIIAHRLNTI
IDCDKILVLDSGRVQEFSSPENLLSNEGSSFSKMVQSTGAANAEYLRSLVLDNKRAKDDS
HHLQGQRKWASSRWAAAAQFALAASLTSSHNDLQSLEIEDDSSILKRTNDAVVTLRSVLE
GKHDKEAESLEEHNISREGWLSSLYRMVEGLAVMSRLARNRMQQPDYNFEGNTFDWDNVE
M

FIGURE 15

gaattcgcggccgccggcgaatttgcactctttacctctctttgactccgtgagattcga
ggattgttagtttcttgtgatgtgtagtcttttgaagcaggggatttttattgtattgagg
aagaagATGGGGTTTGAGCCGTTGGATTGGTATTGCAAGCCGGTGCCGAATGGTGTGTGG
ACTAAAACTGTGGATTATGCGTTTGGTGCATACACGCCTTGTGCTATTGACTCTTTTGTG
CTTGGTATCTCTCATCTGGTTCTGTTGATTCTGTGTCTTTATCGCTTGTGGCTCATCACG
AAGGATCACAAAGTGGATAAGTTCTGCTTGAGGTCTAAATGGTTTAGCTATTTTCTGGCT
CTTTTGGCTGCTTATGCTACTGCGGAGCCTTTGTTTAGATTGGTCATGAGGATCTCTGTT
TTGGATTTGGATGGAGCTGGGTTTCCTCCCTATGAGGCGTTTATGTTGGTCCTTGAGGCT
TTTGCTTGGGGTTCTGCTTTGGTCATGACTGTTGTGGAAACTAAAACGTATATCCATGAA
CTCCGTTGGTATGTCAGATTCGCTGTCATTTATGCTCTTGTGGGAGACATGGTGTTGTTA
AATCTTGTTCTCTCTGTTAAGGAGTACTATGGCAGTTTTAꟻACTGTATCTTTACATAAGC
GAGGTGGCAGTTCAGGTTGCATTTGGAACCCTCTTGTTTGTGTATTTCCCTAATTTGGAC
CCTTACCCTGGTTACACACCAGTTGGGACTGAAAATTCCGAGGATTACGAGTATGAAGAG
CTTCCTGGAGGAGAAAATATATGTCCTGAGAGGCATGCAAATTTATTTGACAGTATCTTC
TTCTCATGGTTGAACCCATTGATGACTCTGGGATCAAAACGACCTCTCACCGAGAAGGAT
GTATGGCATCTGGACACTTGGGATAAAACTGAAACTCTTATGAGGAGCTTCCAGAAGTCC
TGGGATAAGGAACTAGAAAAGCCCAAACCGTGGCTTTTGAGAGCACTGAACAACAGCCTT
GGGGGAAGGTTTTGGTGGGGTGGCTTTTGGAAGATTGGGAATGACTGTTCACAGTTCGTG
GGGCCTCTTCTACTGAATGAGCTCTTAAAGTCAATGCAACTTAATGAACCAGCGTGGATA
GGTTACATCTATGCAATCTCAATCTTTGTTGGAGTGGTATTGGGGGGTTTTATGTGAAGCT
CAGTATTTCCAAAATGTGATGCGTGTTGGTTACCGGCTTAGGTCTGCACTGATTGCTGCT
GTGTTCCGAAAATCTTTGAGGCTAACTAATGAGGGGCGGAAGAAGTTTCAAACAGGAAAA
ATAACAAACTTAATGACTACTGATGCTGAGTCGCTGCAGCAAATCTGCCAATCACTTCAT
ACCATGTGGTCGGCGCCATTTCGTATAATTGTAGCACTGGTTCTCCTCTATCAACAATTG
GGTGTTGCCTCGATCATTGGTGCATTGTTTCTTGTCCTTATGTTCCCCATACAGACTGTT
ATTATAAGCAAAACGCAGAAGTTAACAAAAGAAGGGTTGCAGCGTACTGACAAGAGAATT
GGCCTAATGAATGAGGTTTTAGCGGCAATGGATACAGTGAAGTGTTACGCTTGGGAAAAC
AGTTTTCAGTCCAAGGTTCAAACTGTACGTGATGATGAATTATCTTGGTTCCGGAAAGCA
CAACTCCTGTCAGCGTTCAATATGTTCATACTAAACAGCATCCCTGTCCTCGTGACTGTT
GTTTCATTTGGTGTGTTCTCATTGCTTGGAGGAGATCTGACACCTGCAAGAGCGTTTACG
TCACTCTCTCTATTTTCTGTGCTTCGCTTCCCTTTATTCATGCTTCCAAACATTATAACT
CAGATGGTAAATGCTAATGTATCCTAAACCGTTTGGAGGAGGTACTGTCAACCGAAGAGA
GAGTTCTCTTACCGAATCCTCCCATTGAACCTGGACAGCCAGCTATCTCAATAAGAAATG
GATACTTCTCCTGGGATTCAAAGGCGGATAGGCCAACATTGTCAAACATCAACCTGGACA
TACCTCTTGGCAGCCTAGTTGCGGTAGTTGGCAGCACAGGAGAAGGAAAAACCTCCCTGA
TATCTGCTATGCTTGGGGAACTTCCTGCAAGATCTGATGCGACTGTTACTCTTAGAGGAT
CAGTCGCTTATGTTCCACAAGTTTCATGGATCTTTAACGCAACAGTACGTGACAATATAT
TGTTTGGGGCTCCTTTTGACCAAGAAAAATATGAAAGGGTGATTGATGTGACAGCACTCC
AGCATGACCTTGAGTTACTGCCTGGAGGTGACCTCACGGAGATCGGAGAAAGGGGTGTTA
ACATCAGTGGGGGACAAAAGCAGAGGGTTTCTATGGCTAGGGCCGTTTACTCAAATTCAG
ACGTGTGCATCTTAGATGAACCATTGAGTGCCCTTGATGCGCATGTTGGTCAGCAGGTTT
TTGAAAAATGCATAAAAAGGGAACTAGGGCAGACAACGAGAGTACTTGTTACAAATCAGC
TCCACTTCCTATCACAAGTGGATAAAATCCTACTTGTCCATGAGGGAACAGTAAAAGAGG
AAGGAACATATGAAGAATTATGCCATAGTGGCCCGTTGTTCCCGAGGTTAATGGAAAATG
CAGGGAAGGTTGAAGATTATTCCGAAGAAAATGGAGAAGCTGAAGTACATCAAACATCTG
TAAAACCAGTTGAAAATGGGAACGCTAATAATCTGCAGAAGGATGGAATCGAGACAAAGA
ATTCCAAAGAAGGAAACTCTGTTCTTGTCAAACGAGAAGAACGTGAAACTGGAGTTGTGA
GTTGGAAAGTCCTGGAGAGGTACCAGAATGCACTTGGAGGTGCATGGGTAGTGATGATGC

FIGURE 16A

TCGTTATATGCTACGTCTTGACTCAAGTATTTCGGGTTTCAAGCATCACTTGGTTGAGTG
AGTGGACTGATTCAGGAACCCCAAAGACTCATGGACCCCTATTCTATAATATTGTCTATG
CGCTTCTTTCGTTTGGACAGGTCTCTGTGACATTGATCAATTCATATTGGTTGATTATGT
CCAGTCTATATGCAGCTAAAAAGATGCATGATGCTATGCTTGGTTCCATACTAAGGGCTC
CAATGGTGTTCTTTCAAACCAATCCATTAGGACGGATAATCAATCGATTTGCAAAAGATA
TGGGAGATATTGATCGAACTGTGGCAGTCTTTGTAAACATGTTTATGGGTTCAATCGCAC
AGCTTCTTTCAACTGTTATCTTGATTGGCATTGTCAGCACTCTGTCCCTGTGGGCCATCA
TGCCCCTGTTGGTCGTGTTCTATGGAGCTTATCTGTATTACCAGAACACATCTCGGGAAA
TTAAACGTATGGATTCCACTACAAGATCGCCAGTTTATGCTCAATTTGGTGAGGCATTGA
ATGGACTATCTAGTATCCGTGCTTATAAAGCATATGACAGGATGGCTGAAATTAATGGAA
GGTCAATGGACAATAACATCAGATTCACACTTGTAAACATGGCTGCAAATCGGTGGCTGG
GAATCCGTTTGGAAGTTTTGGGAGGTCTCATGGTTTGGTGGACTGCTTCATTAGCCGTCA
TGCAGAACGGAAAGGCAGCGAACCAACAAGCATATGCATCTACGATGGGTTTGCTTCTCA
GTTATGCGTTAAGCATTACCAGCTCTTTAACAGCTGTACTGAGACTCGCGAGTCTAGCTG
AGAATAGTTTAAACTCGGTTGAGCGTGTTGGAAATTATATCGAGATACCATCAGAGGCTC
CATTGGTCATTGAAAACAACCGTCCACCTCCCGGATGGCCATCATCTGGATCCATAAAAT
TTGAGGATGTTGTTCTTCGTTACCGCCCTGAGTTACCTCCTGTTCTTCATGGAGTTTCGT
TCTTGATTTCTCCAATGGATAAGGTGGGAATTGTTGGGAGGACAGGCGCTGGGAAATCAA
GCCTCTTAAATGCCTTATTCAGGATTGTGGAGCTGGAAAAAGGAAGGATTTTAATTGATG
AATGCGACATTGGAAGATTTGGACTGATGGACCTACGTAAAGTGGTCGGAATTATACCGC
AAGCGCCAGTTCTTTTCTCAGGTACCGTGAGATTCAATCTTGACCCATTTAGTGAACACA
ACGACGCCGATCTCTGGGAATCTCTTGAGAGGGCACACTTGAAAGATACTATCCGCAGAA
ATCCTCTTGGTCTTGATGCTGAGGTAACTGAGGCAGGAGAGAATTTCAGTGTTGGACAGA
GACAGTTGTTGAGTCTTGCACGTGCATTGTTACGAAGATCTAAGATACTTGTTCTTGATG
AAGCAACTGCTGCAGTTGACGTAAGAACTGATGTTCTCATCCAAAAGACCATCCGAGAAG
AATTCAAGTCATGCACAATGCTAATCATCGCTCATCGTCTCAATACTATCATCGACTGTG
ACAAAGTTCTTGTCCTTGATTCTGGAAAAGTTCAGGAATTCAGTTCACCGGAGAATCTTC
TTTCAAATGGAGAAAGTTCTTTCTCGAAGATGGTTCAAAGTACAGGAACTGCAAACGCGG
AGTACTTACGTAGTATAACACTAGAGAACAAACGTACCAGAGAAGCTAACGGTGATGATT
CACAACCTTTAGAAGGTCAAAGGAAATGGCAAGCTTCTTCTCGTTGGGCTGCAGCTGCTC
AATTTGCATTGGCTGTGAGCCtCACTTCATCTCACAACGACCTCCAAAGCCTTGAAATCG
AAGATGATAACAGTATTTTGAAGAAAACAAAGGACGCCGTCGTCACTTTACGCAGTGTCC
TTGAAGGGAAACATGATAAAGAGATTGAAGACTCTCTAAACCAAAGTGACATCTCTAGAG
AGCGTTGGTGGCCATCTCTTTACAAAATGGTCGAAGGGCTTGCCGTGATGAGCAGATTGG
CGAGGAACAGAATGCAACACCCGGATTACAATTTAGAAGGGAAATCGTTTGACTGGGACA
ATGTCGAGATGTAAacgatgaaaggcttacactaatagacctaaaactcccattttgatg
gaacttttatttgtattgcttgggatacacgtaacaaaatgcccattaatcgtggtgtaa
ctatataggctatgcttcttttgggaaaaagagagtttgattacagaggatgtgatgata
acacaattggaattc

FIGURE 16B

gggaggtttggtttttttccctatcaatcgaattccatttcgtgctcgtaacgtggatttttggtaga
tttttttttaggggggatggaaacttgtttattatctatagatgatgattttgttttctccatgagaa
tgtatgctttttaaactttttttttttttgttttttgccttcggagctaactttgggggctggtctcg
gtctctgttttctctccactaaaaagataaaaagcttttgccatctttttttttttttctcaataatc
tatcacatcgtttttttttctttgtttttttctccatttgtcttcattgagttcatagccacataat
tattgatttctttttcttttagtgtttctgttactgatgcgtttcattatttatacttctcacttg
cagattcgaggattgttagtttcttgtgatgtgtagtctttgaagcaggggatttttattgtattg
aggaagaagATGGGGTTTGAGCCGTTGGATTGGTATTGCAAGCCGGTGCCGAATGGTGTGTGGACT
AAAACTGTGGATTATGCGTTTGGTGCATACACGCCTTGTGCTATTGACTCTTTTGTGCTTGGTATC
TCTCATCTGGTTCTGTTGATTCTGTGTCTTTATCGCTTGTGGCTCATCACGAAGGATCACAAAGTG
GATAAGTTCTGCTTGAGGTCTAAATGGTTTAGCTATTTTCTGGCTCTTTTGGCTGCTTATGCTACT
GCGGAGCCTTTGTTTAGATTGGTCATGAGGATCTCTGTTTTGGATTTGGATGGAGCTGGGTTTCCT
CCCTATGAGgtgtgttatcactttgctgtttgttgatgttgttctccttctgtatgttttttcct
gagagatgctgttgtttttgtgctttatttggcagGCGTTTATGTTGGTCCTTGAGGCTTTTGCTTG
GGGTTCTGCTTTGGTCATGACTGTTGTGGAAACTAAAACGTATATCCATGAACTCCGTTGGTATGT
CAGATTCGCTGTCATTTATGCTCTTGTGGGAGACATGGTGTTGTTAAATCTTGTTCTCTCTGTTAA
GGAGTACTATGGCAGgttggtaaatttgcagtctgtatggtttatgcaattttgtttccctggtct
ggcacgatgaacttatatgcgtcatttttttttttgtttttggcagTTTTAAACTGTATCTTTACAT
AAGCGAGGTGGCAGTTCAGgtttgcactttaaaactcctttttgcattctccaaactactctttac
catgtgctgtatctaagtcacactgtaaatgatacaactttgttttttataatgacgttaaggatgg
tttttggatccagGTTGCATTTGGAACCCTCTTGTTTGTGTATTTCCCTAATTTGGACCCTTACCC
TGGTTACACACCAGTTGGGACTGAAAATTCCGAGGATTACGAGTATGAAGAGCTTCCTGGAGGAGA
AAATATATGTCCTGAGAGGCATGCAAATTTATTTGACAgtatgtcactctacacttctcattccct
actttgtttttataggtgcattttctattttaattgtgagaattgccaccgcatcttttatcactt
ttctgcacttactacctatctaagttggttatttatgcagagcttaaatatttccctggaattgta
aattttcttatggagtgctaatacgtagtaggtcattaaaattgtttccgcagagagtagtctata
gtctcttcaaaatttttttttgacttatcctcccgttctccctagaaatgaacttatgatttgtga
ctgtgccgaggttttttgcttagtgatcatcacttcgactaagctgcaacattttatatagtatatt
cgtcaacatttgtcaaactttgactattatgttccttcttacccttgtctttcaacccacagGTAT
CTTCTTCTCATGGTTGAACCCATTGATGACTCTGGGATCAAAACGACCTCTCACCGAGAAGGATGT
ATGGCATCTGGACACTTGGGATAAAAACTGAAACTCTTATGAGGAGgtatattttaataaataacaa
ctgttctcatactgtctatgactggcatggttgcgtgacatatttttatctcattttttagCTTCC
AGAAGTCCTGGGATAAGGAACTAGAAAAGCCCAAACCGTGGCTTTTGAGAGCACTGAACAACAGCC
TTGGGGGAAGgtaaacaaaaacttcttcacagtcatgtgtttttcatctttttgggctttgacatga
tgtgtgatttgtaaaaggaagcatttggttgtaataataaatgcattatgaataactagaagctga
gaaatctgttatggctgtgacttcaagtatgtttttgatgcgtgtcgagttgaataagaaatgtgtt
actttctggttataatctgccatagatactttccatccttatggactgtctgtttctgcattttg
tagGTTTTGGTGGGGTGGCTTTTGGAAGgtactttgtActctttattgtgtttttattctttattc
tgaaacagtcttttccttgtctatttgataatattgatggcttctgaggtcttagttttcctaaat
ggtgtgtttttgtaactgtttaatcttgacatttcaatctaaattgtatcatagATTGGGAATGACT
GTTCACAGTTCGTGGGGCCTCTTCTACTGAATGAGCTCTTAAAGtttgttcctttacttcttttt
accccgtgcacattgtgcttgaacctatttaacacaatgctttgtaattttttccattcacatggat
ctttgagatggattcatattcctactggctcgaataagtgtttaaacgttcttgatagattcaaaa
tcctatcatcctttgaatattatgttctgacgatatctcacaatgtctcctttaactttccgcagT
CAATGCAACTTAATGAACCAGCGTGGATAGGTTACATCTATGCAATCTCAATCTTTGTTGGAGTGg
tatgcaacaaattctcttttttcttcgctgcctttattattctcttgcatggactgcaaaggatatg
aaacaaaaactctactttccttggattctttctttcttgctaggacttcatggtattttttggtct
agagtagatgctacgaattgtaggaccagtttaatttttcttaagctgaaagtaatctctgtgcgat
tcgattgtattagaaaatagcctgattctactcttagagttagttttttttgtttgttaatacatt
tgcatgttgaaaaggtttttgtttaatgtaggtcaaggtgacacttgaccaatggactccttgatcg
cttgatgttgatgttgacattttcagGTATTGGGGGTTTTATGTGAAGCTCAGTATTTCCAAAATG
TGATGCGTGTTGGTTACCGGCTTAGGTCTGCACTGgtaagaaaaagtttcacatgaattatctttt
gctacttagttttttctt

FIGURE 17A

tttgctctgcttctcatgttttgatgcaatacctgtactgttatgtctgttgaaagctatagcaga
tgcttatagattgcttcattctgctgatgaattctcccttaatagATTGCTGCTGTGTTCCGAAAA
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ACTGATGCTGAGTCGCTGCAGgtgtatctttgttacctttactctctttagccttgtctgtttctt
gatataaatttacactgcatagttgtatatctacctcaaaatatgagtcttagatgcaatttacca
agatagtcttttcctgcaactgacgactgaatctgaagcttattctaagattctagaaatcctaa
gagttgtgattacattttcaacaccccttgttctttttgttgccgttgtaggatttgattttccttta
ttagccaataaacctttaattcgcttgatttgtagaaaaaagttacctttgaacagtgcttttatc
taagctcttgcttgaaatcaaagtgtttatctagctgatagctgttcttttttccctaacgtttctc
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AGCACTGGTTCTCCTCTATCAACAATTGGGTGTTGCCTCGATCATTGGTGCATTGTTTCTTGTCCT
TATGTTCCCCATACAGgttcgtatatcttaataattccccattctctttgcgctgtcggttttttt
ttccttttgattgcttatttctcatttgcttttcacaccaatgaaaatgattcatttcctccgttt
atttggttgaaacagACTGTTATTATAAGCAAAACGCAGAAGTTAACAAAAGAAGGGTTGCAGCGT
ACTGACAAGAGAATTGGCCTAATGAATGAGGTTTTAGCGGCAATGGATACAGTGAAgtacgatact
ttggaagcctgaaacctaatatttattttcttgcatagttggaagtttgtggcagtgtttaactat
ctcactaaaccaaaatactgtagGTGTTACGCTTGGGAAAACAGTTTTCAGTCCAAGGTTCAAACT
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acttgtcgctctcctaacggtatgattgtatacaaggacaaatccaagttgctcgtcttttttaaat
gcctttgaccatttttgagaatggtatccatcaatatgtgtttaggcattttctgtactattttcta
gttcattgaacattgattcagttgtttcgggcatgtgtagcagcattcatgcatgatctttaacat
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CTAAACAGCATCCCTGTCCTCGTGACTGTTGTTTCATTTGGTGTGTTCTCATTGCTTGGAGGAGAT
CTGACACCTGCAAGAGCGTTTACGTCACTCTCTCTATTTTCTGTGCTTCGCTTCCCTTTATTCATG
CTTCCAAACATTATAACTCAGgtgatttccttaaaatgtttcttgaaccatgttttcatgtccagt
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TTCAAAGgtcttctttgtctattttatcacatgttcttacttctattagtttctatcattacatat
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AATGCATAAAAAGGGAACTAGGGCAGACAACGAGAGTACTTGTTACAAATCAGCTCCACTTCCTAT
CACAAGTGGATAAAATCCTACTTGTCCATGAGGGAACAGTAAAAGAGGAAGGAACATATGAAGAAT
TATGCCATAGTGGCCCGTTGTTCCCGAGGTTAATGGAAAATGCAGGGAAGGTTGAAGATTATTCCG
AAGAAAATGGAGAAGCTGAAGTACATCAAACATCTGTAAAACCAGTTGAAAATGGGAACGCTAATA
ATCTGCAGAAGGATGGAATCG

FIGURE 17B

AGACAAAGAATTCCAAAGAAGGAAACTCTGTTCTTGTCAAACGAGAAGAACGTGAAACTGGAGTTG
TGAGTTGGAAAGTCCTGGAGAGgtaagttggcattcggatttttgctctttcttgttgtgttgttg
cagtattcctttctatcgacagtggaaatatccgtaaataagacatattctttggtttagagcaat
atgtcaatttatctgtggtgtttctttactacaaaatggatatatattgtttgactcgctctattc
atattcatacaaaatgtatatatattttccgtattaaggttcgtattgtaaagccattgtaataac
ttgtgaggtgtcaccatgttccagGTACCAGAATGCACTTGGAGGTGCATGGGTAGTGATGATGCT
CGTTATATGCTACGTCTTGACTCAAGTATTTCGGGTTTCAAGCATCACTTGGTTGAGTGAGTGGAC
TGATTCAGGAACCCCAAAGACTCATGGACCCCTATTCTATAATATTGTCTATGCGCTTCTTTCGTT
TGGACAGgtatgagttgcatttggcaaatgtttgagtcggtatcttcatgatcggataacaatata
taactgaacattaaaggctgatcagttaagaatatacaccatgtttcttctgcgccaaagtatcga
gcaaacaaaatggaaaataaaaggatacagagagcaaaacgtttattgctaacacgtatttctgcg
ggggtttgtcagGTCTCTGTGACATTGATCAATTCATATTGGTTGATTATGTCCAGTCTATATGCA
GCTAAAAAGATGCATGATGCTATGCTTGGTTCCATACTAAGGGCTCCAATGGTGTTCTTTCAAACC
AATCCATTAGGACGGATAATCAATCGATTTGCAAAAGATATGGGAGATATTGATCGAACTGTGGCA
GTCTTTGTAAACATGTTTATGGGTTCAATCGCACAGCTTCTTTCAACTGTTATCTTGATTGGCATT
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ATGCAGAACGGAAAGGCAGCGAACCAACAAGCATATGCATCTACGATGGGTTTGCTTCTCAGTTAT
GCGTTAAGCATTACCAGCTCTTTAACAGCTGTACTGAGACTCGCGAGTCTAGCTGAGAATAGTTTA
AACTCGGTTGAGCGTGTTGGAAATTATATCGAGATACCATCAGAGGCTCCATTGGTCATTGAAAAC
AACCGTCCACCTCCCGGATGGCCATCATCTGGATCCATAAAATTTGAGGATGTTGTTCTTCGTTAC
CGCCCTGAGTTACCTCCTGTTCTTCATGGAGTTTCGTTCTTGATTTCTCCAATGGATAAGGTGGGA
ATTGTTGGGAGGACAGGCGCTGGGAAATCAAGCCTCTTAAATGCCTTATTCAGGATTGTGGAGCTG
GAAAAAGGAAGGATTTTAATTGATGAATGCGACATTGGAAGATTTGGACTGATGGACCTACGTAAA
GTGGTCGGAATTATACCGCAAGCGCCAGTTCTTTTCTCAGGTACCGTGAGATTCAATCTTGACCCA
TTTAGTGAACACAACGACGCCGATCTCTGGGAATCTCTTGAGAGGGCACACTTGAAAGATACTATC
CGCAGAAATCCTCTTGGTCTTGATGCTGAGgtacttaattaaatatttccatttgggaaagtctca
tgtattcagtaataataactcagtcttttggtcagGTAACTGAGGCAGGAGAGAATTTCAGTGTT
GGACAGAGACAGTTGTTGAGTCTTGCACGTGCATTGTTACGAAGATCTAAGATACTTGTTCTTGAT
GAAGCAACTGCTGCAGTTGACGTAAGAACTGATGTTCTCATCCAAAAGACCATCCGAGAAGAATTC
AAGTCATGCACAATGCTAATCATCGCTCATCGTCTCAATACTATCATCGACTGTGACAAAGTTCTT
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TTACGTAGTATAACACTAGAGAACAAACGTACCAGAGAAGCTAACGGTGATGATTCACAACCTTTA
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AAAACAAAGGACGCCGTCGTCACTTTACGCAGTGTCCTTGAAGGGAAACATGATAAAGAGATTGAA
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Ggtaacgttattcttaagatttctgatacgagtatacgacataaagaattgttgaagtttcttgat
ctaataatttgtgtatatactctcagGGCTTGCCGTGATGAGCAGATTGGCGAGGAACAGAATGCA
ACACCCGGATTACAATTTAGAAGGGAAATCGTTTGACTGGGACAATGTCGAGATGTAAacgatgaa
aggcttacactaatagacctaaaactcccattttgatggaacttttatttgtattgcttgggatac
acgtaacaaaatgcccattaatcgtggtgtaactatataggctatgcttcttttgggaaaaagaga
gtttgattacagaggatgtgatgataacacaattggaattcaaatttgcagcaaaatttgggagaa
aaaaaaaagtcaatgagtgcaacatgcc

FIGURE 17C

aacatggtttcaacttctggacatggacaaccattggacataatttctctcacaggaccatgtttt
gtcattgacattttgcacaaaaatgttctattaaacatatatctataaagaatttgaacaattgtt
aaaa aaacacttaaaatataaattgcaatacaaatttcctttttttt

FIGURE 17D

SUBSTITUTE SHEET (RULE 26)

MGFEPLDWYCKPVPNGVWTKTVDYAFGAYTPCAIDSFVLGISHLVLLILCLYRLWLITKD
HKVDKFCLRSKWFSYFLALLAAYATAEPLFRLVMRISVLDLDGAGFPPYEAFMLVLEAFA
WGSALVMTVVETKTYIHELRWYVRFAVIYALVGDMVLLNLVLSVKEYYGSFKLYLYISEV
AVQVAFGTLLFVYFPNLDPYPGYTPVGTENSEDYEYEELPGGENICPERHANLFDSIFFS
WLNPLMTLGSKRPLTEKDVWHLDTWDKTETLMRSFQKSWDKELEKPKPWLLRALNNSLGG
RFWWGGFWKIGNDCSQFVGPLLLNELLKSMQLNEPAWIGYIYAISIFVGVVLGVLCEAQY
FQNVMRVGYRLRSALIAAVFRKSLRLTNEGRKKFQTGKITNLMTTDAESLQQICQSLHTM
WSAPFRIIVALVLLYQQLGVASIIGALFLVLMFPIQTVIISKTQKLTKEGLQRTDKRIGL
MNEVLAAMDTVKCYAWENSFQSKVQTVRDDELSWFRKAQLLSAFNMFILNSIPVLVTVVS
FGVFSLLGGDLTPARAFTSLSLFSVLRFPLFMLPNIITQMVNANVSLNRLEEVLSTEERV
LLPNPPIEPGQPAISIRNGYFSWDSKADRPTLSNINLDIPLGSLVAVVGSTGEGKTSLIS
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DLELLPGGDLTEIGERGVNISGGQKQRVSMARAVYSNSDVCILDEPLSALDAHVGQQVFE
KCIKRELGQTTRVLVTNQLHFLSQVDKILLVHEGTVKEEGTYEELCHSGPLFPRLMENAG
KVEDYSEENGEAEVHQTSVKPVENGNANNLQKDGIETKNSKEGNSVLVKREERETGVVSW
KVLERYQNALGGAWVVMMLVICYVLTQVFRVSSITWLSEWTDSGTPKTHGPLFYNIVYAL
LSFGQVSVTLINSYWLIMSSLYAAKKMHDAMLGSILRAPMVFFQTNPLGRIINRFAKDMG
DIDRTVAVFVNMFMGSIAQLLSTVILIGIVSTLSLWAIMPLLVVFYGAYLYYQNTSREIK
RMDSTTRSPVYAQFGEALNGLSSIRAYKAYDRMAEINGRSMDNNIRFTLVNMAANRWLGI
RLEVLGGLMVWWTASLAVMQNGKAANQQAYASTMGLLLSYALSITSSLTAVLRLASLAEN
SLNSVERVGNYIEIPSEAPLVIENNRPPPGWPSSGSIKFEDVVLRYRPELPPVLHGVSFL
ISPMDKVGIVGRTGAGKSSLLNALFRIVELEKGRILIDECDIGRFGLMDLRKVVGIIPQA
PVLFSGTVRFNLDPFSEHNDAD   FSLERAHLKDTIRRNPLGLDAEVTEAGENFSVGQRQ
LLSLARALLRRSKILVLDEATAAVDVRTDVLIQKTIREEFKSCTMLIIAHRLNTIIDCDK
VLVLDSGKVQEFSSPENLLSNGESSFSKMVQSTGTANAEYLRSITLENKRTREANGDDSQ
PLEGQRKWQASSRWAAAAQFALAVSLTSSHNDLQSLEIEDDNSILKKTKDAVVTLRSVLE
GKHDKEIEDSLNQSDISRERWWPSLYKMVEGLAVMSRLARNRMQHPDYNLEGKSFDWDNV
EM

FIGURE 18

Fig. 19A

DNP-GS uptake
(nmol/mg)

pYES3-AtMRP1

PYES3

Time (min)

Fig. 19B

DNP-GS uptake
(nmol/mg/10 min)

[DNP-GS] (µM)

Fig. 20A

DNP-GS uptake (nmol/mg/10 min)

pYES3-AtMRP1

pYES3

[Vanadate] (µM)

Fig. 20B

DNP-GS uptake (nmol/mg/10 min)

[Vanadate] (µM)

Fig. 21

AtMRP1

AtMRP2

ScYCF1

HmMRP1

RtCMOAT1

Amino acid residue

Fig. 22
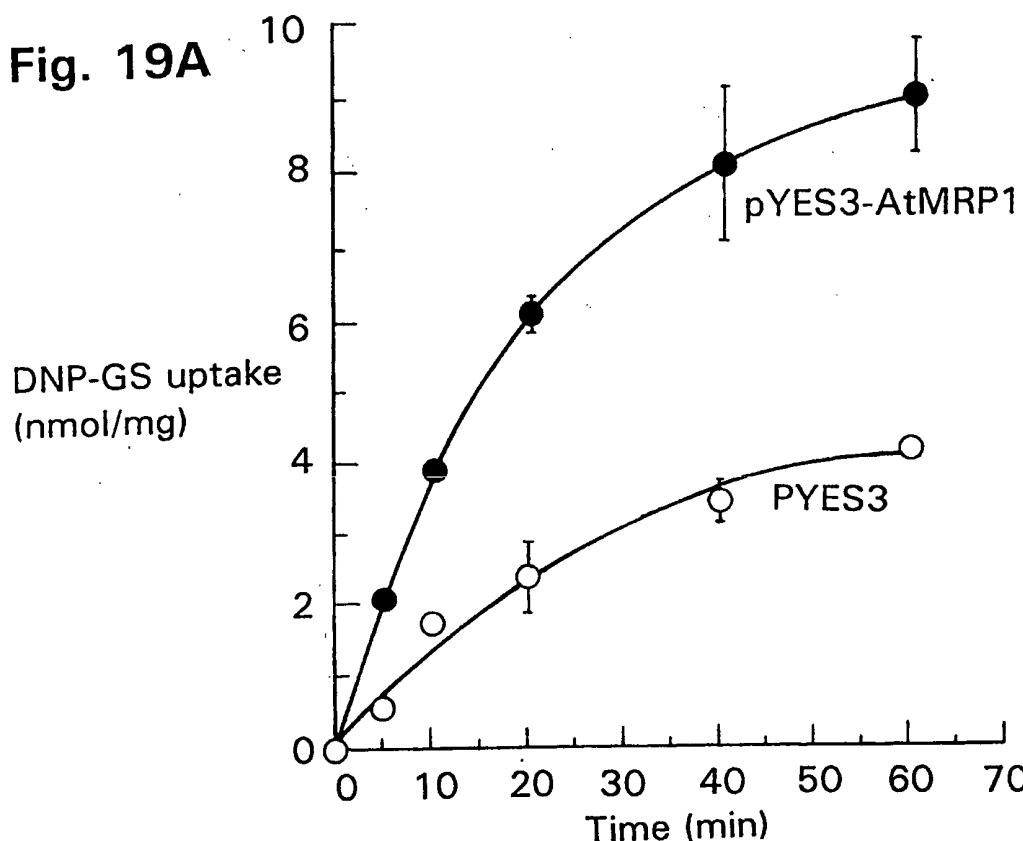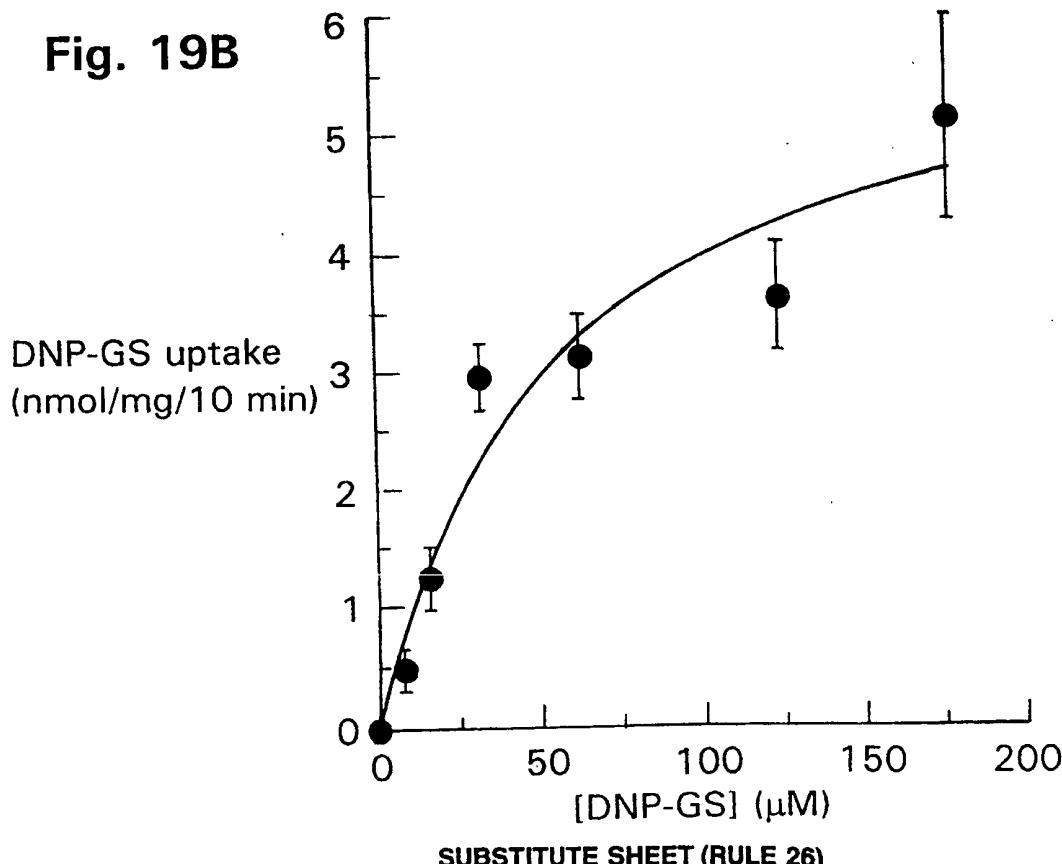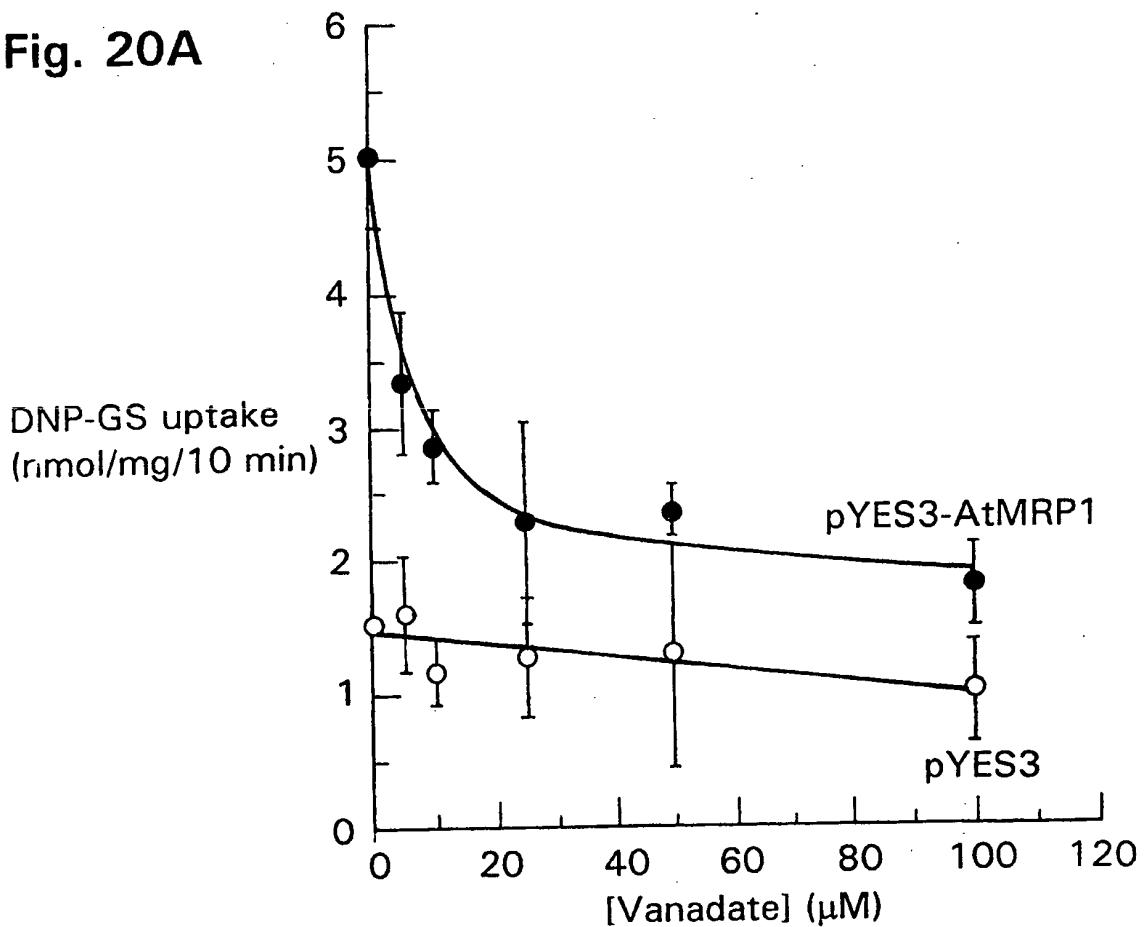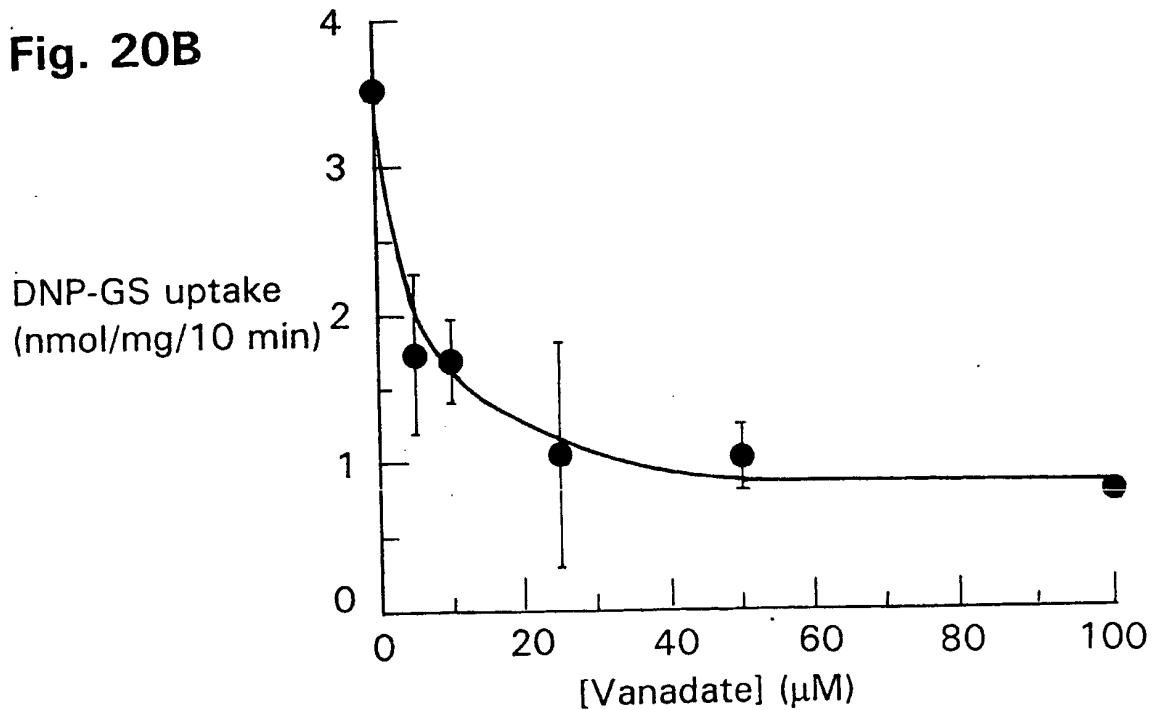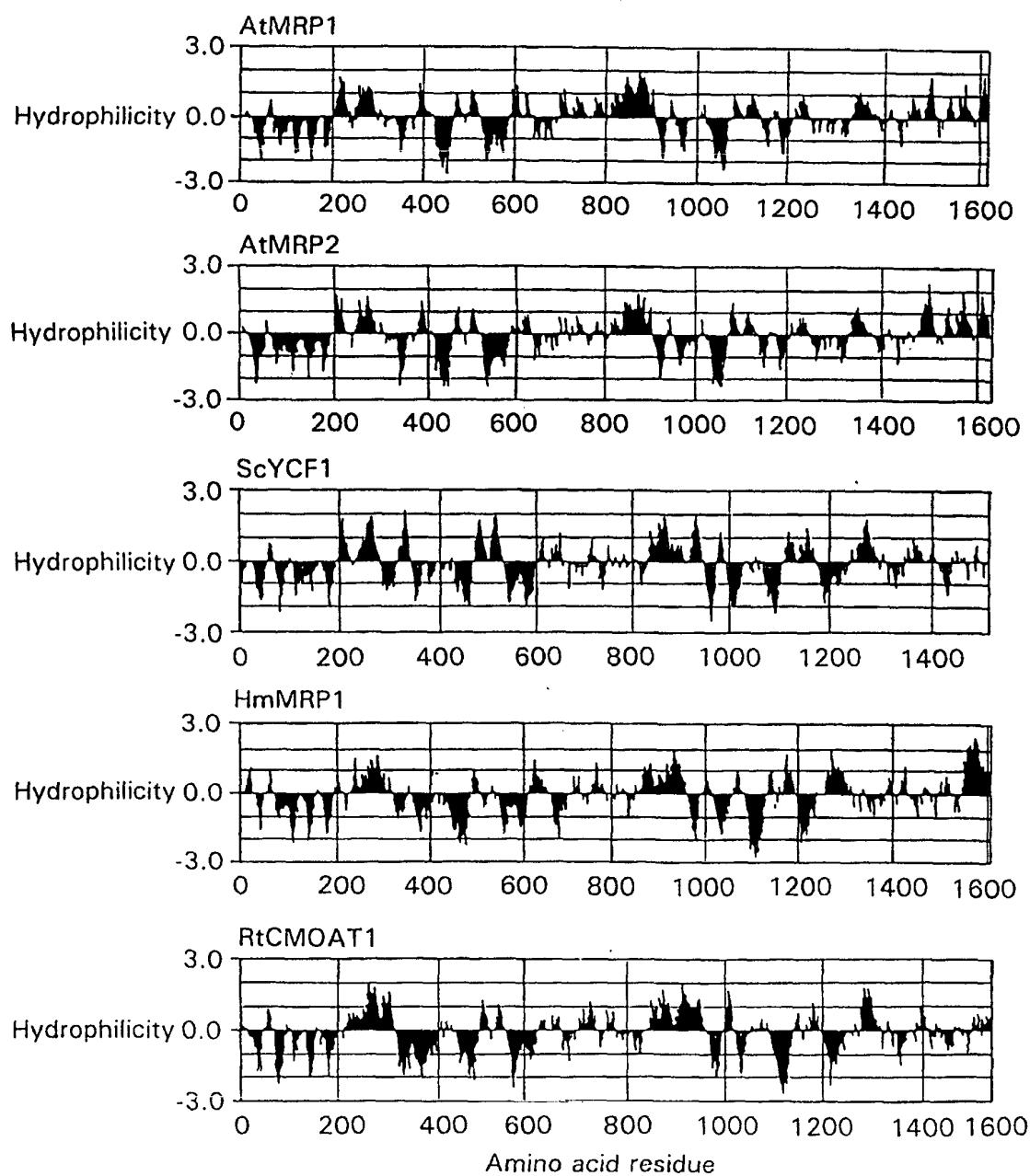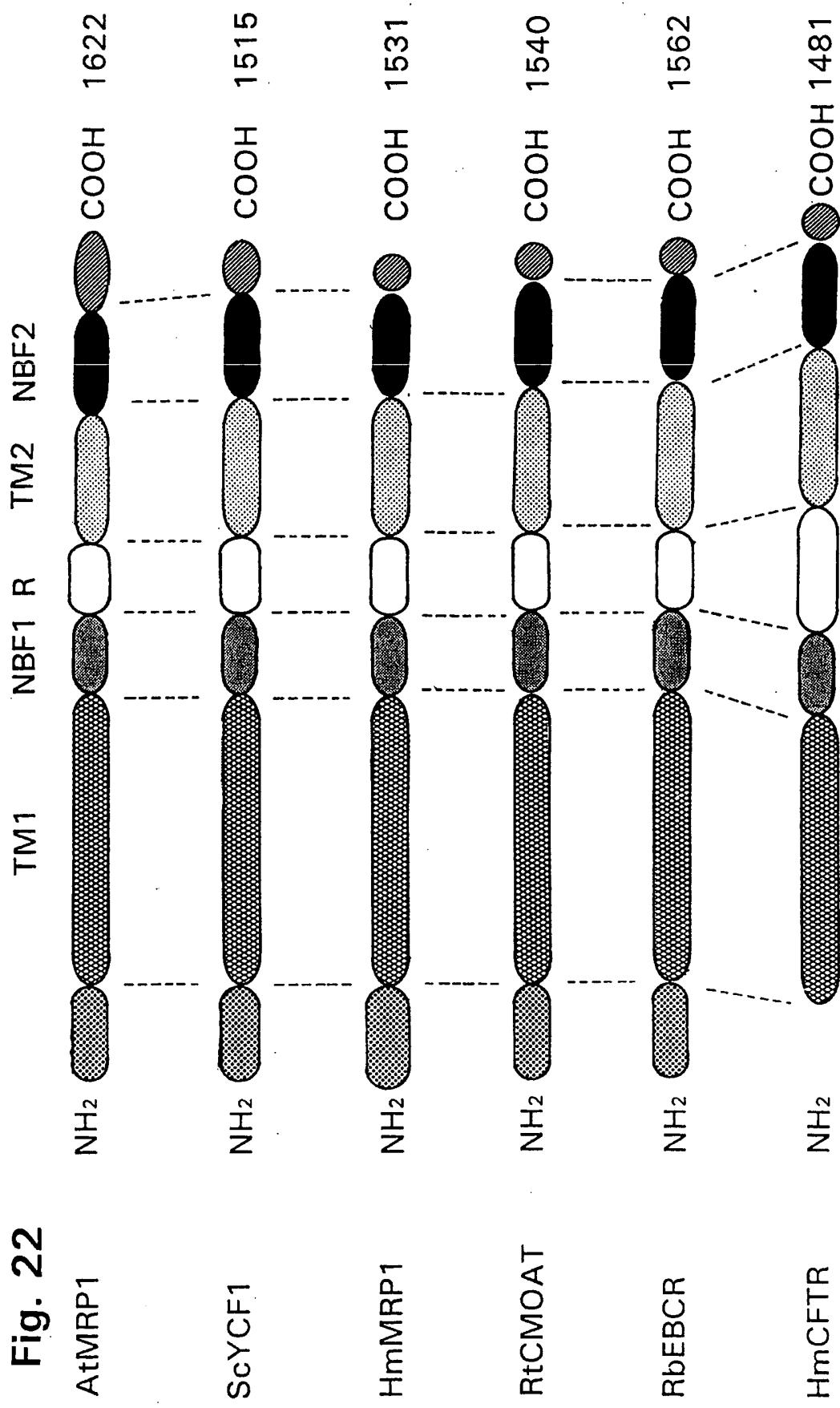
AtMRP1 Promoter Sequence: 1253 bp

Contains:

2 Myb recognition sequences (**atatcgtta** and **agttagtt**).
1 xenobiotic regulatory element (GCGTG).
1 antioxidant response element (**Gtgacaaa**).

NB: Several "RNA instability determinants" (*ATTTA*) found but these usually located in the 3'-UTRs of genes? May simply be reflection of AT-richness of sequence.

ttcactttgtcctttttttcttaacatctactttgtcatcagcaaattatctgtaaataa
gatagggtttatgcttattgctacaatgaacctaatcctatgatgtgtattgcaatttgcaa
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ttacaaaacagagcatcaaaaatcaaaagaacatatcgcataatcgaactatgctaatacct
ctcctcaatctttgttgttgttatattcaagtagcttattcttttgtttttattttacgatta
gatttctctaga*ATTTAATTTA*tatt*ATTTA*atcatacttgatcaaggtttgtagcttaatc
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tcgtatagtgtaagcagtgacgcatcc*ATTTA*tcatcatctccttataaatctcgaatctgc
cacagagaga<u>GCGTG</u>**tgacaaa**atgagttcataagattccgttatcgtcttcctgattcctc
caaatctccgg

FIGURE 23

AtMRP2 Promoter Sequence: 1368 bp

Contains:

1 bZIP recognition sequence (**cacgtg**).
Xenobiotic regulatory element not found.
Antioxidant response element not found.

NB: Several "RNA instability determinants" (_ATTTA_) found but these usually located in the 3'-UTRs of genes? May simply be reflection of AT-richness of sequence.

```
aaacaattggtgtattttgaattttttcatgcaacgcacgtgaacagcttaattgcttgatt
ggaaacaaacctttttagaattcattaatcagttttaggtgttttggaaaattaacgaact
atagtggagattaattaattttatattagtctttttttagtacacaaatcgaagtttcctag
atttttttcaaagttgaaaataatattgataatATTTAtcaacaatgaatctacaaaaacat
aattttttttgccaaacaaataacaccgaaacaagattcattcactattttttggtttaaaaa
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```

FIGURE 24

Fig. 25

Fig. 26
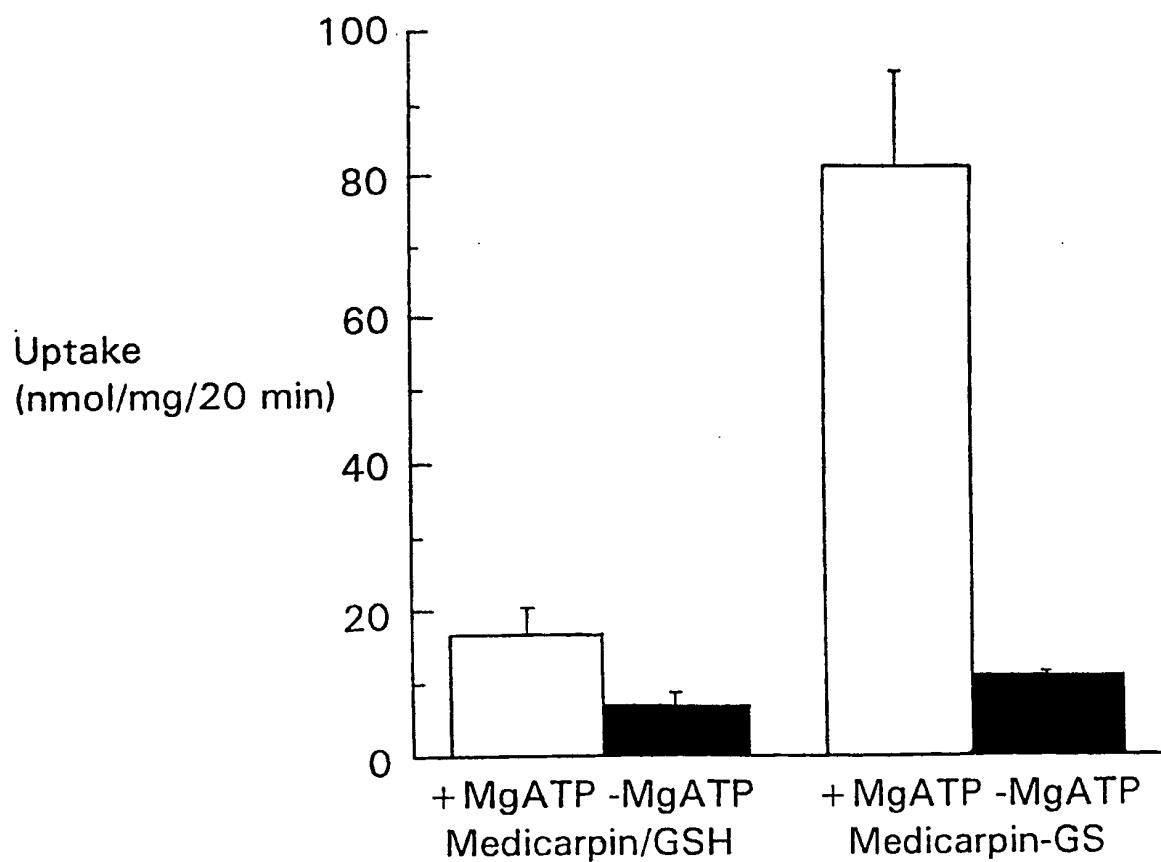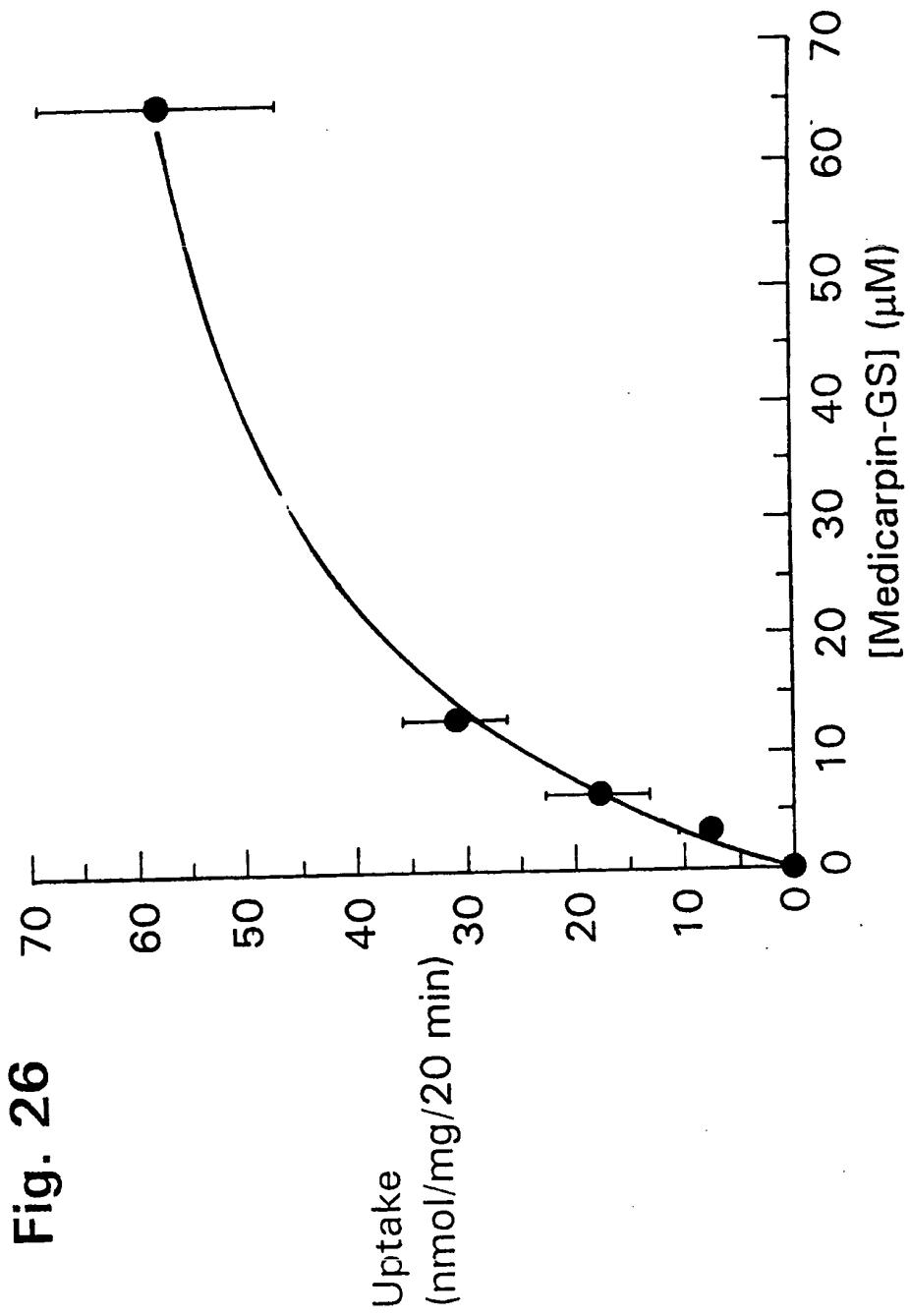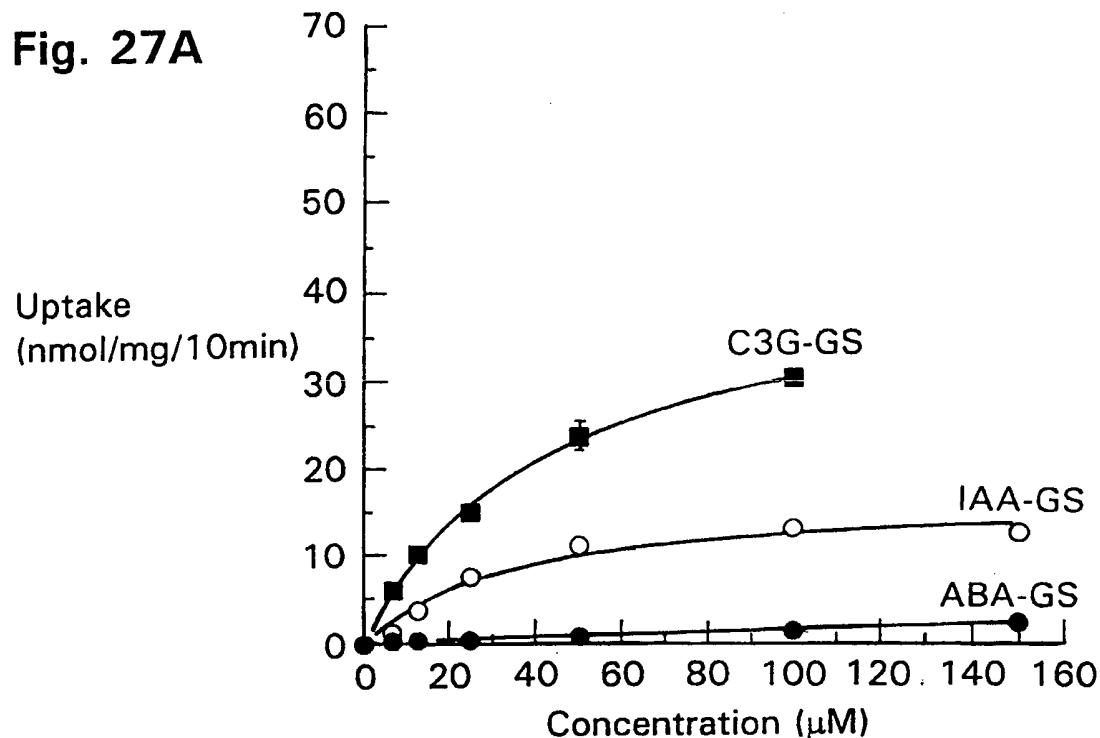
Fig. 27A

Fig. 27B
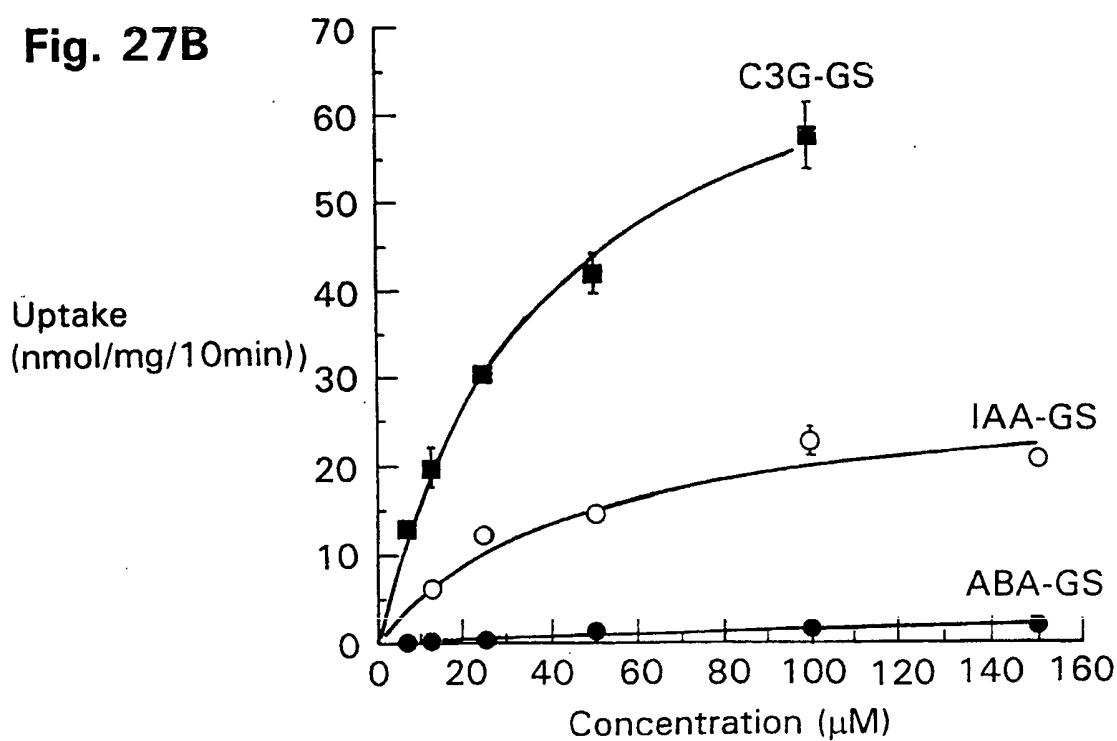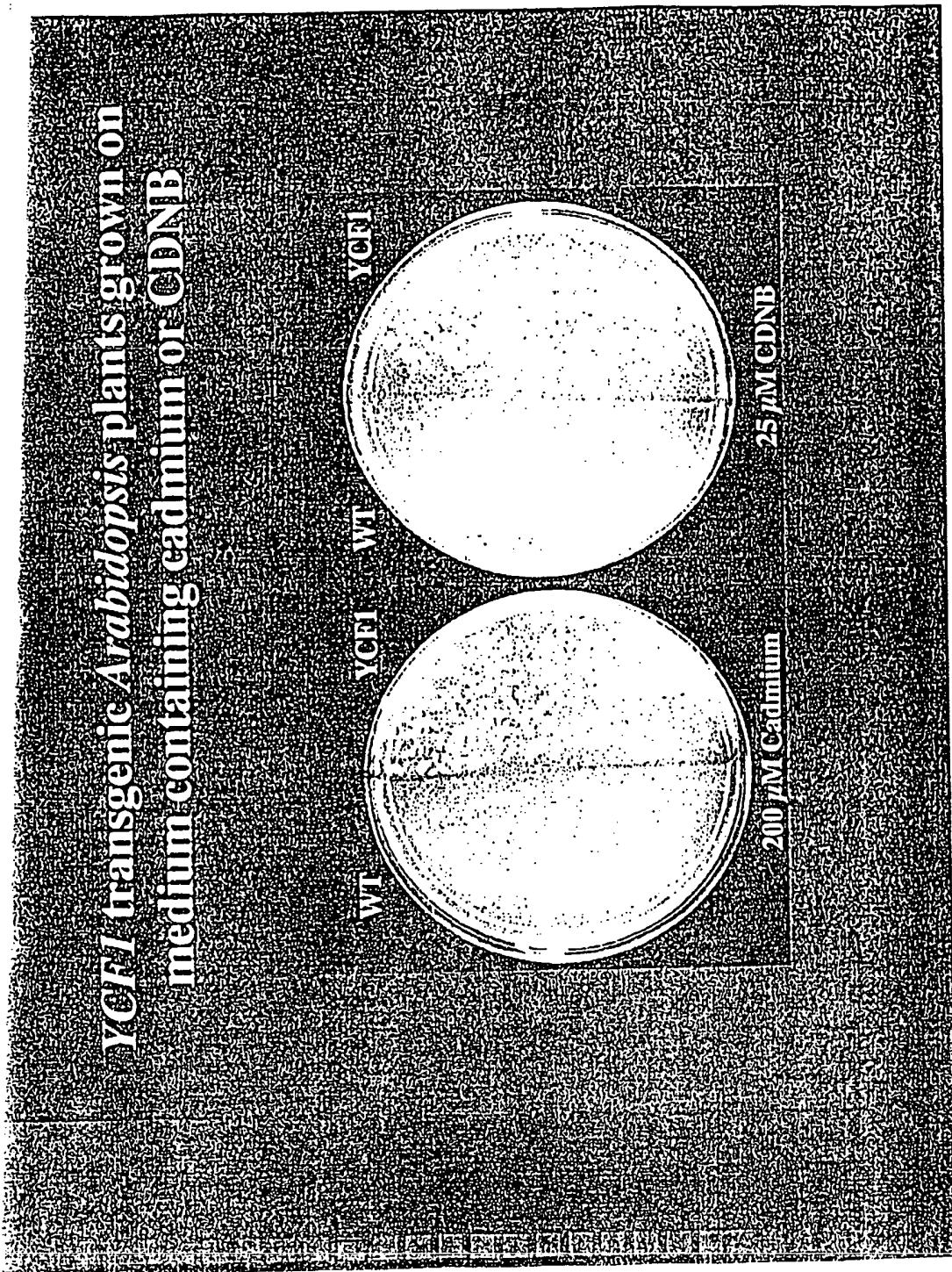
FIGURE 28

INTERNATIONAL SEARCH REPORT

A.   CLASSIFICATION OF SUBJECT MATTER

IPC(6)   :Please See Extra Sheet.

US CL   :Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B.   FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

  U.S. :   536/23.6, 23.1, 24.1; 530/350, 387.9; 435/252.3, 419, 172.3, 6; 800/205

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

  APS, DIALOG - Biotech files

C.   DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
T	ISHIKAWA, T. et al. The GS-X Pump in Plant, Yeast, and Animal Cells: Structure, Function, and Gene Expression. Bioscience Reports. 1997. Vol 17, No. 2, pages 189-207, see entire document.	1-27
A	LI, Z.S. et al. Magnesium Adenosine 5'-Triphosphate-Energized Transport of Glutathione-S-Conjugates by Plant Vacuolar Membrane Vesicles. Plant Physiol., 1995, Vol 107, pages 1257-1268, see entire document.	1-27

[X]   Further documents are listed in the continuation of Box C.   [ ]   See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance		
"E"	earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
26 JANUARY 1998	2 3 FEB 1998
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer ELIZABETH C. KEMMERER
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Form PCT/ISA/210 (second sheet)(July 1992)★

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
T	LI, Z.S. et al. A new pathway for vacuolar cadmium sequestration in Saccharomyces cerevisiae: YCF1-catalyzed transport of bis(glutathionato)cadmium. Proc. Natl. Acad. Sci. USA, January 1997, Vol 94, pages 42-47, see entire document.	1-27
T	LU, Y.P. et al. AtMRP1 gene of Arabidopsis encodes a glutathione S-conjugate pump: Isolation and functional definition of a plant ATP-binding cassette transporter gene. Proc. Natl. Acad. Sci. USA. July 1997, Vol 94, pages 8243-8248, see entire document.	1-27
A	MARTINOLA, E. et al. ATP-dependent glutathione S-conjugate 'export' pump in the vacuolar membrane of plants. Nature, 15 July 1993, Vol 364, pages 247-9249, see entire document.	1-27
A	LI, Z.S. et al. The Yeast Cadmium Factor Protein (YCF1) Is a Vacuolar Glutathione S-Conjugate Pump. J. Biol. Chem., 15 March 1996, Vol 271, No. 11, pages 6509-6517, see entire document.	1-27

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

A01H 5/00; C07K 14/415, 16/16; C12N 1/13, 1/21, 5/10, 15/29, 15/64, 15/82

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

536/23.6, 23.1, 24.1; 530/350, 387.9; 435/252.3, 419, 172.3, 6; 800/205